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Plasma Prothrombin Time in Skin Diseases

II. Clinical Investigation on Plasma Prothrombin Time

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Materials and method of plasma prothrombin time determination is quite similar to those of the preceeding article.

1) Response of Plasma Prothrombin Time (P.P.T.) during Treatments

P.P.T. is determined more than twice—before and during treatment in 73 skin diseases. Prompt recovery of protracted P.P.T., especially of 12.5% quite corresponding clinical improvement, is observed in 18 cases i.e. 3 cases each of contact dermatitis and urticaria chronica, 2 cases each of drug exanthema and erythema nodosum, one case each of ectodermose érosive pluriorificielle, toxic eruption and urticaria perstans papulosa and also in one case each of extensive burns, bullosis mechanica senilis, acrodermatitis continua Hallopeau and hydroa vacciniformis.

Elongation of protracted P.P.T., especially of 12.5% is observed in 2 cases pemphigus vulgaris and one case Hodgkin's disease and all the patients died short after the tests.

No recovery of P.P.T. was found in other almost unimproved cases such as eczema chronicum, purpura, eczema seborrhoicum, cutaneous tumor, pityriasis rubra Hebra and fungus infection.

In three cases of erythematodes discoides chronicus P.P.T. was determined at least once a month for over 10 months; Case 1) Female, 30 years, protraction of 12.5% P.P.T. was noticed at first after 2 months oral administration of Atebrin (daily 0.2 g.), then whole P.P.T. tended to protract one month later. Recovery was noticed at first in 12.5% P.P.T. following interruption of the drug and intravenous administration of amino acids. Case 2) Male, 61 years, protracted both P.P.T. at his admittance was recovered by 28.5 g. of Chloromycetin and 10 g. of streptomycin. Atebrin administration (0.2 g. daily for 40 days) protracted both P.P.T. and interruption of the drug and liver extract injection normalized both P.P.T. Case 3) Female, 27 years, protracted both P.P.T. at her con-

sultation tended to normal by 30 times of Mycillin injections. ACTH, cortisone and daily 0.3 g. Atebrin for 13 days with vitamin C mixed with 20 cc. of 20% glucose solution did not influence both P.P.T. It seems important that in all cases variations of P.P.T., especially of 12.5%, is always precursory to urobilinogen in urine and Takata reaction.

2) Relationship between P.P.T. and Other Clinical Tests

Urobilinogen in urine was tested comparatively in 370 cases. In cases of normal P.P.T. (102 cases) urobilinogen was negative in 86 cases (84%) but in cases of protracted P.P.T. (268 cases) urobilinogen was positive in 74 cases (28%). Takata reaction was tested comparatively in 120 cases and no significant finding was obtained.

Influence of vitamin K upon P.P.T. has been examined mostly in the field of internal medicine and detailed variations of P.P.T. after vitamin K injection were reported by many investigators. The author tried this in 18 cases of skin diseases and 3 cases of liver diseases as the control. P.P.T. is determined before and 24 hours after intravenous injection of vitamin K (5 cc. contains 100 mg. $V.K_s$—sodium bisulfite) dissolved in 20 cc. 20% glucose solution because of severe headache. Mean value of the difference of whole P.P.T. before and after the injection is—1.5 seconds and that of 12.5% P.P.T. is—20.2 seconds. In 4 cases of skin diseases which present decrease of both P.P.T. 24 hours after the injection no positive urine urobilinogen and Takata reactions were found, but in other cases P.P.T. tended to protract and especially 4 cases presenting above 20.0 seconds rose in their 12.5% P.P.T. 24 hours after the injection urobilinogen in urine and Takata reaction were always positive.

No constant variations of P.P.T. before and 1, 3 and 5 hours after intravenous injection of 20 cc. 10% sodium thiosulfite aqueous solution were found in 11 cases.

P.P.T. and sedimentation rate of erythrocyte were examined comparatively in 248 cases but no definite conclusion is obtained. The fact almost corresponds to, the resuls of Yokota *et al.*[1]

Also no intimate relationship between P.P.T. and eosinophilic response following epinephrine injection in 108 skin disease cases was found but 4 hours after subcutaneous injection of 0.5 cc. of 1 :1000 epinephrine to 17 skin disease patients, 7 cases presented reduced P.P.T. after injection and in 10 cases P.P.T. tended to protract.

Aschner's phenomenon and P.P.T. were tested in 368 cases but no difinite relationship was found.

3) Relationship between P.P.T. and Reticuloendothelialsystem (R. E.S.)

Yamagata *et al.*[2][3] stated in their animal experiment that functional trialw ith the injection of congored solution presented less variation of

P.P.T. than that of congored index (Adler-Reimann's method) and concluded that congored index indicated the function of R.E.S. and P.P.T. presented the functional condition of the liver cell. Also they have noticed that albumin increases and globulin decreases following the stimulation of R.E.S. and that albumin increases but globulin presents no constant results in experimental injuries of the liver.

The author tried the comparative tests of P.P.T. and congored index (C.I.) by Adler-Reiman's method (normal range in 50.0~70.0 in health controls) in 90 skin diseases and found that the C.I. is abnormal in 60 cases (67.5%) which presented protraction of P.P.T. Also γ-globulin was determined by Tiselius' method simultaneously in 57 cases and protraction of P.P.T. accompanied with increased γ-globulin was found in 37 cases (64.9%), but no intimate finding was obtained in a comparative determination of total serum protein (86 cases). According to above mentioned reasons, the author made an experiment of P.P.T. with congored injection in 13 cases of skin diseases. P.P.T. is determined before and 1, 3, 5 hours after congored injection (pro kilo 0.2 cc. of one per cent aqueous congored solution). By the results of three hours after the injection the tested patients were separated into two groups, one of which (7 cases) presents increase of P.P.T. and other group (6 cases) presents decrease of P.P.T. (mean values of variation in both groups are $\pm$2.0 sec. in whole and $\pm$15.5 sec. in 12.5 % plasma). In the former, increased C.I. (1 hour after the injection), negative urine urobilinogen and decreased albumin-globulin ratio (before the injection) were found. In the latter, however, normal C.I., positive urine urobilinogen and normal or increased A/G. ratio were shown.

To find out the influence of congored injection upon serum protein fraction, A/G is determined 1, 3, 8, 12 and 24 hours after the injection of congored solution in two skin disease patients presenting both abnormal C.I. and A/G (0.61 and 0.76) before the injection and the latter also shows disturbed liver function. Variation of A/G after the injection is 0.97 (1 h.), 0.92 (3 hrs.), 0.84 (8 hrs.), 0.90 (12 hrs.), 0.63 (24 hrs.), in the former and 1.20, 0.98, 1.08, 1.12, 0.83 respectively in the latter.

Therefore the author concludes that the variation of P.P.T. after the injection of congored solution chiefly depend upon the function of liver cell and also partly upon the condition of R.E.S., especially the alternation of serum protein fraction.

Conclusion

Plasma prothrombin time is determined on whole and 12.5% saline diluted plasma during treatment and is studied comparatively with certain clinical tests in skin disease patients.

1. Plasma prothrombin time of 12.5% saline diluted plasma is more sensitive indicator of prothrombin activity than that of whole plasma.

2. Plasma prothrombin time is considered to be the method of examining the multiple functions of liver cell which varies from urobilinogen test in urine, Takata reaction and sedimentation rate of erythrocyte. The results of test depend chiefly upon the condition of function of the liver cell but partly upon that of reticuloendothelial system.

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Studies on Body Water in Man

I. Total Body Water in Normal Subjects and Edematous Patients

(Directed by Assistant-Professor, K. Itahara)

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Body water occupies about 70%[1] of body weight and is the greatest component of the living organism. All activities of every cell proceed in the medium of " body water ", which is considered as the basis of metabolism. Edema and dehydration, the most important of all clinical problems, represent abnormal accumulation and loss of water in tissues, respectively. In consideration of the importance of body water, methods for its measurement have been studied. The first estimate of body water content was accomplished by desiccation of cadavers. By the desiccation procedure Bischoff[2] reported in 1863 that the total body water in man was 58.5%, and recently Mitchell[3] reported that it was 67.8% of the body weight. But this procedure cannot be employed in living subjects. A satisfactory method for the measurement of the total body water in vivo has long been sought. In 1934 Hevesy[4] first estimated the total body water in living man by use of heavy water (deuterium oxide). Heavy water is an ideal substance for the measurement of the total body water, but the costliness of the substance and the complicated apparatus for its measurement make its use inconvenient. Potassium,[5] thiourea[6] and sulfanilamide[7] were also employed for this purpose, but these are not satisfactory in that they do not distribute uniformly throughout the body water. On the other hand the total body water can be calculated from the specific gravity of the body.[8] However, the specific gravity method is labourious and is not easily applicable to patients.

In 1949 Soberman and his co-workers[9] found that antipyrine satisfies the following conditions necessary for the measuremnt of total body water: (1) uniform and rapid distribution throughout the body water; (2) nontoxicity; (3) slow transformation and excretion; (4) accurate and convenient estimation of its concentration in plasma. So, they employed antipyrine for the measurement of the total body water in man, and showed

that the results obtained by the volume of distribution of antipyrine agreed well with the body water content determined with heavy water.

The total body water in living subjects has been studied in American and Europeans. In this report it was estimated in normal subjects and edematous patients of Japanese by the antipyrine method of Soberman[9], and the variation of the total body water in lean and obese subjects was examined.

Experimental

Methods

After a control sample of blood was drawn in postabsorptive state, 50 cc. of 2% (or 3% in edematous patients) antipyrine solution was injected intravenously. Blood samples were drawn at the 2nd, the 3rd and the 5th hours (or at the 3rd, the 4th and the 6th hours) subsequently. The concentration of antipyrine in plasma was determined at the wavelength of 350 mμ in a Beckman's spectrophotometer by the method of Brodie.[10] When the logarithm of the plasma concentration of antipyrine was plotted against time on semilogarithmic paper, the time-concentration curve yielded a straight line. The concentration at the time of injection was calculated by extrapolating this straight line back to zero time. The calculation of the total body water was made as follows:

$$\text{Total body water} = \frac{\text{amount of antipyrine injected}}{\text{concentration of antipyrine in plasma}}$$

Results

The total body water was determined in normal subjects and hospital patients clinically free from cardiovascular or renal disease, 12 males and 11 females, and in 17 edematous patients.

Normal subjects. As presented in Table I, the total body water of normal males averaged 32.6 *l.* (range 29.4 to 38.6 *l.*) or 56.4% of body weight (range 39.6 to 72.6%), and as presented in Table II the total body water of normal females averaged 27.7 *l.* (range 25.0 to 30.7 *l.*) or 48.6% of body weight (range 35.0 to 61.3%). Its percentage to body weight was higher in males than females, and showed a marked individual variability in both sexes: it was high in lean subjects and low in obese subjects. For example, the highest value of 72.6% was obtained in a very lean man (Height: 157.0 cm., Weight: 43.0 kg.) (No. 1 in Table I), and the lowest value of 35% in a very obese woman (Height: 154.5 cm., Weight: 74.5 kg.) (No. 2 in Table II). This individual variability of body water is due to an inverse relationship of the percentage of body water to the percentage of body fat[11]—the more the fat the less the water. Now, there

TABLE I

Total Body Water in Normal Males

Subject Number	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water l.	Total body water / Body weight %	Total body water / Standard body weight %
No. 1	53	157.0	43.0	54.5	31.2	72.6	57.2
No. 2	29	162.0	49.5	56.7	30.6	61.8	54.0
No. 3	17	162.0	54.0	51.9	29.4	54.4	56.6
No. 4	40	161.5	53.7	57.3	31.8	59.2	55.5
No. 5	22	163.5	61.0	57.1	29.5	48.4	51.6
No. 6	58	168.0	86.2	61.9	34.1	39.6	55.1
No. 7	41	161.0	57.0	56.8	30.3	53.2	53.3
No. 8	34	175.0	62.0	65.0	38.6	62.3	59.4
No. 9	19	174.7	61.8	61.0	33.1	53.6	54.3
No. 10	34	168.4	53.1	60.8	34.2	64.5	56.3
No. 11	50	162.0	67.1	57.6	35.7	53.2	61.9
No. 12	22	165.2	61.3	58.0	33.6	54.8	57.9
Average					32.6	56.4	56.1
Range					29.4~38.6	39.6~72.6	51.6~61.9

must be a state of moderate nutrition, neither lean nor obese, for each individual—that is the standard body weight for his age and height. Since the percentage of the body water depends largely upon the body fat content as presented above, the body water expressed in the percentage of his standard body weight instead of his actual body weight for each individual will be considerably corrected in its individual variability. For this reason the body water was calculated in the percentage of the standard body weight for each individual, which was obtained from the standard age-height-weight table published by the Ministry of Welfare.[12] As shown in the last column of Table I and II, its range contracted to between 51.6 and 61.9% in normal males, and to between 50.9 and 61.4% of the standard body weight in normal females, showing a far less conspicious variability than the percentage to their actual body weight. For example, the value of 72.6% of his actual body weight decreased to 57.2% of his standard body weight in the case No. 1 in Table I, and 35.0% increased to 50.9% of her standard body weight in the case No. 2 in Table II. Typical examples are illustrated in Fig. 1. The average of the percentage to their standard body weight was 56.1% in normal males and 55.0% in normal females (Tables I and II). It is interesting that the difference between the average of male and female becomes smaller by calculating

TABLE II

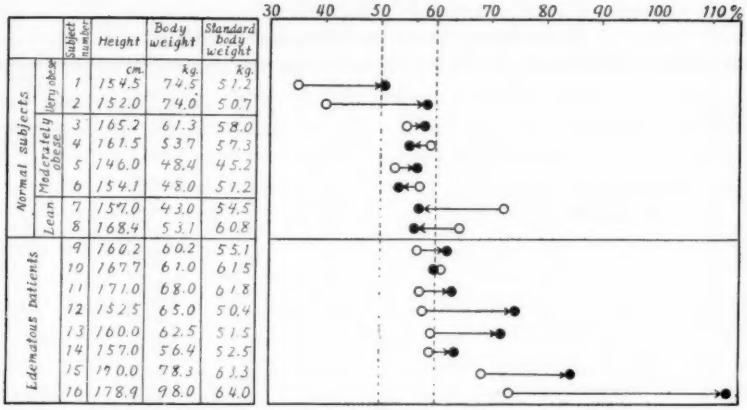
Total Body Water in Normal Females

Subject Number	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water *l.*	Total body water / Body weight %	Total body water / Standard body weight %
No. 1	35	145.5	52.8	47.8	25.1	47.5	52.5
No. 2	28	154.5	74.5	51.2	26.1	35.0	50.9
No. 3,a	35	152.0	74.0	50.7	29.7	40.1	58.6
„ b	„	„	*58.6	„	*26.4	*45.1	*52.1
No. 4	17	155.4	47.8	48.8	25.0	52.3	51.2
No. 5	27	150.6	72.3	50.0	30.7	42.5	61.4
No. 6	19	160.0	54.2	52.1	27.8	51.3	53.4
No. 7	35	153.0	45.5	51.2	27.9	61.3	54.5
No. 8	19	164.5	53.4	56.2	30.5	57.1	54.3
No. 9	22	154.1	48.0	51.2	27.4	57.1	53.5
No. 10	19	153.0	77.0	50.3	29.4	38.2	58.4
No. 11	32	146.0	48.4	45.2	25.6	52.9	56.6
Average					27.7	48.6	55.0
Range					25.0～30.7	35.0～61.3	50.9～61.4

* Values obtained following decrease of body weight on a low calorie diet and by administration of thyreoidin, and excluded in computing the average.

the percentage by the standard body weight than by the actual body weight. A very obese woman (Height: 152.0 cm., Weight: 74.0 kg.) (No. 3 in Table II) lost 15.4 kg. in body weight on a low calorie diet and by administration of thyreoidin (a preparation of thyroid gland), but the reduction of body water was only 3.3 *l.* This indicates that body fat is greatly responsible for the variety of body weight in normal subjects.

Edematous patients. The results obtained with 17 edematous patients are summarized in Table III. The total body water averaged 40.2 *l.* (range 31.3 to 72.2 *l.*) or 62.3% of body weight (range 56.8 to 73.6%). It was higher on the average in edematous patients than in normal subjects. Individually speaking, however, the borderline between the edematous patients and the normal subjects was not obvious: not a few of patients had a lower value than lean normal subjects in spite of obvious edema. For example, 7 of 17 patients had values below 60% of their body weight. But, when the total body water was expressed by its percentage to their standard body weight, the edematous patients were clearly differentiated from the normal subjects: the total body water averaged 70.6% of the standard body weight (range 59.6 to 112.8%) in edematous patients,



		Subject number	Height	Body weight	Standard body weight
			cm.	kg.	kg.
Normal subjects	Very obese	1	154.5	74.5	51.2
		2	152.0	74.0	50.7
	Moderately obese	3	165.2	61.3	58.0
		4	161.5	53.7	57.3
		5	146.0	48.4	45.2
		6	154.1	48.0	51.2
	Lean	7	157.0	43.0	54.5
		8	168.4	53.1	60.8
Edematous patients		9	160.2	60.2	55.1
		10	167.7	61.0	61.5
		11	171.0	68.0	61.8
		12	152.5	65.0	50.4
		13	160.0	62.5	51.5
		14	157.0	56.4	52.5
		15	170.0	78.3	63.3
		16	178.9	98.0	64.0

Fig. 1. Percentage of total body water to body weight (○) and to standard body weight (●).

exceeding 60% in most of them (the last column in Table III). Typical examples are illustrated in Fig. 1. In a markedly edematous patient of chronic nephritis with ascites (No. 16 in Table III) the total body water was 112.8% of the standard body weight. This value above 100% is not unreasonable because it only indicates that the weight of his total body water has increased beyond his standard body weight. In No. 5, 9 and 13 in Table III, who recovered from edema by treatment, the loss of body water (12.8, 8.1 and 8.8 *l.*) was almost paralleled to the reduction of body weight (14.0, 10.5 and 9.8 kg.).

Discussion

Since Hevesy[4] first estimated the total body water in living man, it has been studied by other workers. The total body water as estimated by use of heavy water was reported to be 63% by Hevesy,[4] to be 72.8% by Moore[13], to be 61.8% on the average (range 55.9 to 70.2%) in normal males and 51.9% on the average (range 45.6 to 59.9%) in normal females by Schloerb[14], and 60% of body weight on the average (range 42 to 71%) in normal males by Hardy.[15] On the other hand Soberman and his co-workers[9] employed antipyrine for the measurement of total body water and obtained a value of 54.5% of body weight on the average (range 50.4 to 57.9%) in normal males, and they showed that the values agreed well with those obtained by heavy water method. Since then, the total body water estimated by antipyrine method was reported to be 53% on the average (range 40 to 68%) in normal males and 45% on the average

TABLE III

Total Body Water in Edematous Patients

Subject Number	Diagnosis	Sex	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water *l.*	Total body water / Body weight %	Total body water / Standard body weight %
No. 1	Congestive heart failure	M	55	167.7	61.0	61.5	37.2	61.0	60.5
No. 2	"	M	46	163.0	62.0	58.8	35.6	57.4	60.5
No. 3	"	F	55	160.2	60.2	55.1	34.2	56.8	62.1
No. 4	"	F	33	151.5	51.5	50.6	31.3	60.8	61.9
No. 5,a	"	F	53	150.2	62.0	50.2	39.9	64.4	79.5
" b	"	"	"	"	*48.0	"	*27.1	*56.5	*53.9
No. 6	Pericarditis	M	60	170.0	78.3	63.3	53.6	68.4	84.6
No. 7	Chronic nephritis	M	19	160.2	68.5	57.5	48.8	71.2	84.8
No. 8	"	M	30	165.0	60.0	58.5	36.1	60.1	61.7
No. 9,a	"	M	21	165.0	62.0	58.0	41.4	66.8	71.4
" b	"	"	"	"	*51.5	"	*33.3	*64.7	*57.4
No. 10	"	M	20	171.0	68.0	61.8	39.0	57.4	63.1
N0. 11	"	M	25	167.0	61.3	59.2	35.6	58.1	60.1
No. 12	Nephrosis	F	21	152.5	65.0	50.4	37.6	57.8	74.6
No. 13,a	"	M	17	160.0	62.5	51.5	37.1	59.4	72.0
" b	"	"	"	"	*52.7	"	*28.3	*53.7	*55.0
No. 14	Chronic nephritis	M	26	159.9	53.4	55.5	33.1	61.9	59.6
No. 15	"	M	35	160.1	58.0	55.9	37.9	65.3	67.7
No. 16	"	M	22	178.9	98.0	64.0	72.2	73.6	112.8
No. 17	"	F	26	157.0	56.4	52.5	33.4	59.2	63.6
Average							40.2	62.3	70.6
Range							31.3～72.2	56.8～73.6	59.6～112.8

* Values obtained after disappearance of edema, and not included in computing the average.

(range 30 to 53%) in normal females by Steele[11], and to be 61.1% of body weight on the average (range 43.0 to 72.9%) in normal males by Osserman.[16] As presented above, the total body water ranges widely from about 30 to 70% of body weight, and its values obtained by these workers are not always in accord with one another in the average value. The total body water obtained in this study was 56.4% on the average (range 39.6 to 72.6%) in normal males and 48.6% of body weight on the average (range 35.0 to 61.3%) in normal females.

Behnke[17] estimated the specific gravity of the body in normal males, and Rathbun[18] devised a formula in experiments of animals, by which

body fat can be calculated from the specific gravity of the body. Pace[19] found that the body water occupies 72.4% of lean body mass or fat-free tissue. Applying this principle to the human body, Messinger[8] and Osserman[16] calculated body fat by specific gravity method. Simultaneously they determined the total body water with antipyrine and they showed that the proportion of body water bears an inverse relationship to the proportion of body fat. From this finding it is easily understood that the percentage of the total body water to the body weight obtained in this study was high in lean subjects and low in obese subjects. The inconstancy in the results of estimation of the total body water obtained by other workers and the author is supposed to be due to the selection of subjects, but most of the average values in these results are below 60% of body weight. The generally used value of 70% of body weight[1] is applicable only to very lean subjects such as No. 1 in Table I. In edematous patients the total body water and its percentage to body weight were higher on the average than in normal subjects. Individually speaking, however, edematous patients were not always differentiated from normal subjects in the percentage of the body water to the body weight. Steele[11] also reported that not a few of markedly edematous patients fell within the normal range for body water, and he pointed out that measurement of the total body water alone is of little help in bringing to light abnormal accumulation of fluid because of the marked individual variability in the proportion of the body water.

At this point the percentage of the total body water to the standard body weight was proposed as a criterion for abnormal accumulation of body water in this study. Since the percentage of total body water to body weight depends largely upon fat content as stated above, the total body water expressed in the percentage to the standard body weight for each individual will be considerably corrected in its individual variability. In fact, the data presented here showed that the percentage of the total body water to the standard body weight in normal subjects was far less remarkable in its variability than its percentage to the actual body weight, and its range contracted to between 50 and 60% of the standard body weight. Soberman[9] stated that an average man, neither lean nor obese, contains about 20% fat when body fat is calculated from the data of the body specific gravity of normal males obtained by Welham.[20] The body water of an average man, calculated on the assumption that fat-free tissue contains 72.4% water, would be 58% of the body weight.

$$(100-20)\times\frac{72.4}{100}=57.9\%$$

From the above data and considerations, about 60% of the standard body weight seems to be the upper limit of the body water in normal

subjects, while the total body water exceeds 60% of their standard body weight in most of edematous patients. Thus, when the total body water is expressed in the percentage of the standard body weight, a line of demarcation can be drawn between normal subjects and edematous patients, which is not possible when the actual body weight is used in the estimation.

Summary

1. The total body water was estimated by the antipyrine method in normal subjects and edematous patients.

2. The total body water averaged 56.4% (range 39.6 to 72.6%) in 12 normal males, and averaged 48.6% of their body weight (range 35.0 to 61.3%) in 11 normal females. It presented a remarkable individual variability: it was high in lean subjects and low in obese subjects.

3. Edematous patients were not always differentiated from normal subjects in the percentage of the total body water to their body weight. But a borderline can be drawn between normal subjects and edematous patients by expressing the total body water in the percentage to their standard body weight: About 60% of the standard body weight is the upper limit of the total body water in normal subjects, while the total body water exceeded 60% of the standard body weight in most of the edematous patients.

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Muscle Membrane Potential of the Free Wall of Dog's Ventricle

By

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Hitherto the transmembrane resting and action potential of the excised ventricular muscle of the dog was studied almost exclusively either at the false tendon (Purkinje fiber)[1] or the papillary muscle[2] because of the experimental conveniences, e.g., smallness of its mechanical activity.

However, the impalement of membrane and recording of potential at the subendocardial layer throughout the free ventricular wall or the septum was found to be quite easily and successfully carried out when the microelectrode having the tip appropriate in shape and size was used. The typical pattern of the action potential from the subendocardial cell is, as illustrated in Fig. 1 A, provided with a manifest initial spike followed by a slightly upward convex plateau. Interesting to note is the fact that such a pattern of action potential is intermediate between those of the Purkinje fiber (Fig. 1 B) and the papillary muscle (Fig. 1 C).

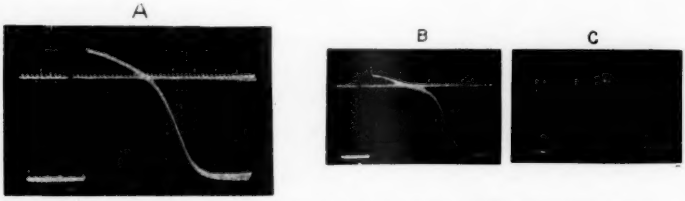

Fig. 1. Transmembrane potential of ventricular free wall (A) of the dog as contrasted to those of Purkinje fiber (B) and papillary muscle (C). Extracellular potential is at the level of the line of time scale (10 and 50 ms). -100 mV is indicated by a dot on the bottom.

On the average, the resting potential was 90±3.9 mV (s. D.), the overshoot was 44±5.3 mV (s. D.) and the maximum velocity of depolarization was 530 ±180 V/sec. (s. D.).

Muscles of deeper or subepicardial layer, on the other hand, showed the action potential quite similar to the papillary muscle. The details of the results will be reported elsewhere.

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Studies on Body Water in Man

II. Body Water Compartments in Normal Subjects and Edematous Patients

(Directed by Assistant-Professor, K. Itahara)

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In a previous report[1] the total body water in man was studied. It is important for the understanding of water metabolism to know not only the total body water but also its distribution. The complete solution of the problem of body water distribution is not yet accomplished. The body water is distributed in two main compartments—extra- and intracellular spaces. The total body water is indicated by the volume of distribution of antipyrine.[2] When the total body water and the extracellular fluid are determined, the intracellular fluid can be calculated as the difference between them. For the measurement of the extracellular fluid sodium thiocyanate is widely used because of its convenience, but it has a disadvantage in that it penetrates into cells.[3][4] Although the volume of distribution of inulin is accepted to be most reliable for the measurement of the extracellular fluid, the long time required for its uniform distribution makes its use inconvenient in the presence of edema.[5] Since Newman and his associates[6] found that the glomerular filtration rate of mannitol is related to the volume of the extracellular fluid, mannitol has come into use for its measurement.

In this report the following were examined in normal subjects and edematous patients with cardiac or renal disease by simultaneous determination of the spaces of antipyrine, thiocyanate and mannitol: 1) extra- and intracellular fluid; 2) the relation between the spaces of thiocyanate and mannitol which are both used in measuring the extracellular space; 3) the shift of the body water accompanying alleviation of edema.

Experimental

Methods

After a control blood sample was drawn in a postabsorptive state, 50 cc. of 2% (or 3% in edematous patients) antipyrine solution and 10 cc.

of 5% sodium thiocyanate solution were intravenously injected simultaneously. Following the injection, 100 cc. of 15% mannitol solution was intravenously injected for the purpose of priming injection. Thereafter, a constant infusion of 3% mannitol solution obtained by diluting 15% mannitol solution with 0.9% NaCl solution was continued at a rate of about 4 cc./min. for one hour (or two hours in edematous patients).

1) Calculation of antipyrine space (total body water) was made by the method of Soberman[2] as previously reported.[1]

2) Calculation of thiocyanate space. One hour (two hours in edematous patients) after the injection a blood sample was drawn and urine was collected. The concentration of thiocyanate in serum and urine was determined at the wave-length of 480 mμ in a Beckman's spectrophotometer by the method of Gregersen.[3] The calculation of thiocyanate space was made by the following formula.

$$\text{Thiocyanate space} = \frac{\text{amount injected} - \text{amount excreted in urine}}{\text{concentration in serum}}$$

3) Calculation of mannitol space (extracellular fluid). Schwartz[7] and his associates developed the Newman's[6] principle and proposed a constant infusion technique without collection of urine for the measurement of extracellular fluid. When the constant infusion of a substance is continued until the substance is uniformly distributed and its concentration in plasma arrives at an equilibrium, the fall of the concentration of the substance in plasma bears the following relation to its volume of distribution:

$$V_s = \frac{Tc\ (t'-t)}{\ln P - \ln P'}$$

V_s: the volume of distribution of the substance.
T_c: total plasma clearance of the substance.
P and P′: the concentration in plasma at the time of t and t′ after the cessation of infusion, respectively.

A blood sample was drawn immediately before the cessation of infusion, and two blood samples at an interval of 15 minutes after it. The concentration of mannitol in plasma was determined at the wavelength of 570 mμ in a Beckman's spectrophotometer by the method of Corcoran.[8] The calculation of the mannitol space was made by the above formula of Schwartz.[7]

Results

In this report the extracellular fluid was represented by the mannitol space and the intracellular fluid was calculated as the difference between the antipyrine space (total body water) and the mannitol space.

Normal subjects. The results obtained with 9 normal subjects are

summarized in Table I. The extracellular fluid averaged 10.8 *l.* (range 9.2 to 13.7 *l.*), 19.3% of the body weight (range 14.2 to 24.2%), or 35.2% of the total body water (range 32.1 to 39.8%). The intracellular fluid averaged 20.1 *l.* (range 17.3 to 24.9 *l.*), 35.7% of the body weight (range 24.0 to 40.3%), or 64.6% of the total body water (range 60.2 to 67.8%). Both the extra- and intracellular fluids showed a considerable individual variability in their percentage to the body weight: they were high in lean subjects and low in obese subjects. But their percentage to the total body water showed less variability. When the extra- and the intracellular fluids were expressed in the percentage to the standard body weight[1] their individual variability became smaller: the former ranged from 17.0 to 21.7% (19.5% on the average) and the latter from 33.8 to 39.3% of the standard body weight (36.0% on the average). The thiocyanate space was larger in all cases than the mannitol space: the thiocyanate-mannitol space ratio averaged 1.18 (range 1.13 to 1.22). It is supposed that thiocyanate penetrates into cells and consequently its volume of distribution presents a larger value than the volume of distribution of mannitol.

Edematous patients with congestive heart failure. The results obtained with 5 patients are summarized in Table II. The extracellular fluid averaged 14.5 *l.* (range 13.2 to 17.2 *l.*), 24.5% of the body weight (range 21.9 to 27.7%), or 40.8% of the total body water (range 38.6 to 43.2%). It is known from the above data that the extracellular fluid in cardiac edema was larger than the normal. Its percentage to the standard body weight averaged 26.5% (range 23.8 to 34.3%), showing its increase more distinctly. The thiocyanate-mannitol space ratio was higher in all patients than the normal, averaging 1.42 (range 1.35 to 1.51). This indicates that more thiocyanate penetrates into cells in these cases than in a normal state, suggesting that cellular permeability to thiocyanate was increased. The intracellular fluid averaged 21.1 *l.* (range 17.8 to 22.7 *l.*), 35.4% of the body weight (range 34.5 to 36.6%), or 59.1% of the total body water (range 56.8 to 61.4%). Its percentage to the body weight and the total body water was lower than the normal on the average. However, it can not be immediately concluded from this low value that the intracellular fluid was decreased, because the percentage of the intracellular fluid to the body weight and the total body water may be lower than the normal, even if the intracellular fluid is increased, when the body water is further increased in the extracellular fluid than in the intracellular fluid. It seems reasonable to use the percentage to the standard body weight as a criterion for abnormal accumulation or loss of intracellular fluid as is the case with the total body water previously reported.[1] The percentage of intracellular fluid to the standard body weight average 38.3% (range 35.2 to 45.2%) in edematous patients with congestive heart failure. It was

TABLE
Distribution of Body Water

Subject Number	Sex	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water *l.*	Extracellular		
							Thiocyanate space *l.*	Mannitol space *l.*	Mannitol space / Body weight %
No. 1	M	34	168.4	53.1	60.8	34.2	14.5	12.8	24.2
No. 2	F	19	164.5	53.4	56.2	30.5	12.9	10.6	19.8
No. 3	M	34	175.0	62.0	65.0	38.6	16.6	13.7	22.1
No. 4	F	22	154.1	48.0	51.2	27.4	12.1	10.1	21.0
No. 5	M	22	163.5	61.0	57.1	29.5	11.2	9.7	15.9
No. 6	F	35	153.0	45.5	51.2	27.9	11.8	10.0	22.0
No. 7	M	22	165.2	61.3	58.0	33.6	12.9	10.8	17.6
No. 8	F	19	153.0	77.0	50.3	29.4	13.1	10.9	14.2
No. 9	F	19	160.0	54.2	52.1	27.8	10.7	9.2	17.0
Average						30.9	12.8	10.8	19.3
Range						27.4～38.6	10.7～16.6	9.2～13.7	14.2～24.2

TABLE
Distribution of Body Water

Subject Number	Diagnosis	Sex	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water *l.*	Extracellular	
								Thiocyanate space *l.*	Mannitol space *l.*
No. 1	Congestive heart failure	M	55	167.7	61.0	61.5	37.2	21.4	14.9
No. 2	"	M	46	163.0	62.0	58.8	35.6	19.7	14.0
No. 3	"	F	55	160.2	60.2	55.1	34.2	19.9	13.2
No. 4	"	F	33	151.5	51.5	50.6	31.3	18.3	13.5
No. 5,a	"	F	53	150.2	62.0	50.2	39.9	24.4	17.2
" b	"	"	"	"	*48.0	"	*27.1	*12.6	* 9.1
Average							35.6	20.7	14.5
Range							31.3～39.9	18.3～24.4	13.2～17.2

* Values obtained after disappearance of edema by administration of mercurial

somewhat higher in 4 of 5 patients than the normal. In these cases the intracellular fluid is considered to be slightly increased. No. 5 in Table

I

in Normal Subjects

fluid		Intracellular fluid (I.C.F.)				
Mannitol space / Standard body weight %	Mannitol space / Total body water %	I.C.F. *l.*	I.C.F. / Body weight %	I.C.F. / Standard body weight %	I.C.F. / Total body water %	Thiocyanate space / Mannitol space
21.1	37.4	21.4	40.3	35.2	62.6	1.13
18.9	34.7	19.9	37.3	35.4	65.3	1.22
21.1	35.6	24.9	40.1	38.3	64.4	1.21
19.8	39.8	17.3	36.0	33.8	60.2	1.20
17.0	32.9	19.8	32.5	34.7	67.2	1.15
19.5	35.8	17.9	39.3	35.0	64.2	1.18
18.6	32.1	22.8	37.2	39.3	67.8	1.20
21.7	37.1	18.5	24.0	36.8	62.9	1.20
17.7	33.2	18.6	34.3	35.5	66.8	1.16
19.5	35.2	20.1	35.7	36.0	64.6	1.18
17.0～21.7	32.1～39.8	17.3～24.9	24.0～40.3	33.8～39.3	60.2～67.8	1.13～1.22

II

in Cadiac Edema

fluid			Intracellular fluid (I.C.F.)				
Mannitol space / Body weight %	Mannitol space / Standard body weight %	Mannitol space / Total body water %	I.C.F. *l.*	I.C.F. / Body weight %	I.C.F. / Standard body weight %	I.C.F. / Total body water %	Thiocyanate space / Mannitol space
24.4	24.2	40.1	22.3	36.5	36.3	59.9	1.44
22.6	23.8	39.3	21.6	34.8	36.7	60.7	1.41
21.9	23.9	38.6	21.0	34.9	38.1	61.4	1.51
26.2	26.6	43.1	17.8	34.5	35.2	56.9	1.35
27.7	34.3	43.2	22.7	36.6	45.2	56.8	1.42
*18.9	*18.1	*33.5	*18.0	*37.5	*35.8	*66.4	*1.38
24.5	26.5	40.8	21.1	35.4	38.3	59.1	1.42
21.9～27.7	23.8～34.3	38.6～43.2	17.8～22.7	34.5～36.6	35.2～45.2	56.8～61.4	1.35～1.51

diuretics, and excluded in computing the average.

II recovered from edema by administration of mercurial diretics, losing 14.0 kg. in body weight and 12.8 *l.* in body water: 8.1 *l.* was lost from the

extracellular and 4.7 *l.* from the intracellular fluids. Though the extracellular fluid returned to normal after the disappearance of edema in this case, the thiocyanate-mannitol space ratio did not return to normal, showing a higher value of 1.38. It is suggested from this observation that the increased cellular permeability to thiocyanate persists even after the disappearance of edema.

TABLE

Distribution of Body

Subject Number	Diagnosis	Sex	Age year	Height cm.	Body weight kg.	Standard body weight kg.	Total body water *l.*	Extracellular	
								Thiocyanate space *l.*	Mannitol space *l.*
No. 1	Chronic nephritis	M	19	160.2	68.5	57.5	48.8	31.2	23.2
No. 2	"	M	30	165.0	60.0	58.5	36.1	17.9	12.3
No. 3,a	"	M	21	165.1	62.0	58.0	41.4	30.2	18.1
" b	"	"	"	"	*51.5	"	*33.3	*15.4	*11.2
No. 4	"	M	20	171.0	68.0	61.8	39.0	21.4	11.6
No. 5	"	M	25	166.9	61.3	59.2	35.6	21.2	10.2
No. 6	Nephrosis	F	21	152.5	65.0	50.4	37.6	29.7	21.0
No. 7,a	"	M	17	160.2	62.5	51.5	37.1	32.6	19.9
" b	"	"	"	"	*52.7	"	*28.3	*16.2	*12.0
Average							39.3	26.3	16.6
Range							35.6~48.8	17.9~32.6	10.2~23.2

* Values obtained after disappearance of edema by administration of cation ex-

Edematous patients with chronic nephritis or nephrosis. The results obtained with 7 patients are summarized in Table III. The extracellular fluid averaged 16.6 *l.* (range 10.2 to 23.2 *l.*), 25.9% of the body weiglt (range 16.6 to 33.8%), 29.8% of the standard body weight (range 17.2 to 41.7%), or 41.9% of the total body water (range 28.7 to 55.9%). These data indicate that the extracellular fluid was more abundant than the normal in cases of renal edema except in two patients in the alleviating stadium of edema (No. 4 and 5 in Table III). The thiocyanate-mannitol space ratio was higher than the normal in all patients, averaging 1.63 (range 1.34 to 2.08). The intracellular fluid averaged 22.7 *l.* (range 16.6 to 27.4 *l.*), 35.6% of the body weight (range 25.5 to 41.5%), or 39.8% of the standard body weight (range 32.9 to 44.5%). A somewhat higher value of its percentage to the body weight and the standard body weight than the

normal indicates that the intracellular fluid is also slightly increased in renal edema with the excetpion of nephrosis (No. 6 and 7 in Table III). The percentage of the intracellular fluid to the total body water was lower than the normal in four of seven patients, averaging 58.1% (range 44.1 to 71.3%). In nephrosis (No. 6 and 7 in Table III) the increase of extracellular fluid was remarkable, but the intracellular fluid rather decreased.

III

Water in Renal Edema

fluid			Intracellular fluid (I.C.F.)				
Mannitol space / Body weight %	Mannitol space / Standard body weight %	Mannitol space / Total body water %	I.C.F. *l.*	I.C.F. / Body weight %	I.C.F. / Standard body weight %	I.C.F. / Total body water %	Thiocyanate space / Mannitol space
33.8	40.3	47.5	25.6	37.3	44.5	52.4	1.34
20.5	21.0	34.1	23.8	39.6	40.7	65.9	1.45
29.2	31.2	43.7	23.3	37.6	40.2	56.3	1.66
*21.3	*19.3	*33.6	*22.1	*42.9	*38.1	*66.3	*1.37
17.2	18.8	29.8	27.4	40.1	44.2	70.2	1.84
16.6	17.2	28.7	25.4	41.5	42.9	71.3	2.08
32.3	41.7	55.9	16.6	25.5	32.9	44.1	1.41
31.8	38.6	53.7	17.2	27.6	33.4	46.3	1.64
*22.7	*23.3	*42.4	*16.3	*30.9	*31.6	*57.6	*1.35
25.9	29.8	41.9	22.7	35.6	39.8	58.1	1.63
16.6～33.8	17.2～41.7	28.7～55.9	16.6～27.4	25.5～41.5	32.9～44.5	44.1～71.3	1.34～2.08

change resin.

On the other hand, the intracellular fluid increased though the extracellular fluid was normal in No. 4 and 5 of Table III, who were in the alleviating stadium of edema on a low salt diet and by administration of Theoharn (Triaminotriazin). This suggests that the edema fluid is at first lost from the extracellular fluid in recovery from edema. No. 3b and 7b in Table III recovered from edema by administration of cation exchange resin, losing 8.1 *l.* and 8.8 *l.* in body water—6.9 *l.* and 7.9 *l.* from the extracellular fluid, respectively. In these two cases the thiocyanate-mannitol space ratio was higher than the normal even after the disappearance of edema, standing at 1.37 and 1.35, respectively.

Discussion

The extracellular fluid represented by inulin space is approximately

15% of the body weight.[9][10] The extracellular fluid represented by mannitol space was reported to be 17.7% by Newman[6], to be 17.3% by Schwartz[7], to be 16% by Dominguez[11], to be 21.2% by Elkinton[12] and to be 19.6% of the body weight by Abe.[13] The value obtained in this study was 19.3% of the body weight on the average (range 14.2 to 24.2%). It presented a considerable individual variability; it was high in lean subjects and low in obese subjects. Kaltreider[14] reported similar results, and Hardy[15] pointed out that the extracellular fluid is more closely correlated with the total body water and lean body mass than the body weight. In this report the extracellular fluid of normal subjects presented a less variation in its percentage to the total body water than in its percentage to the body weight. When the extracellular fluid was represented by its percentage to the standard body weight, its variability became smaller, and its range from 14.2 to 24.2% of the body weight contracted to between 17.0 and 21.7% of the standard body weight. Also in the percentage of intracullular fluid to the standard body weight similar findings were obtained, and its range from 24.0 to 40.3% of the body weight contracted to between 33.8 and 39.3% of the standard body weight. Thus the extra- and the intracellular fluids were more closely correlated with the total body water and the standard body weight than the actual body weight.

Berger and associates[5] and Deane[16] studied the distribution of the body water by simultaneous determination of the total body water and the extracellular fluid. According to them, about 15% of the body weight or 35% of the body water is in the extracellular space, and about 40% of the body weight or 70% of the body water is in the intracellular space. The data presented here indicate that about 20% of the body weight or 35% of the body water is in the extracellular space and about 35% of the body weight or 65% of the body water is in the intracellular space. The difference between these two data is due to the use of mannitol in this study instead of inulin used by Berger[5] and Deane[16] in estimation of the extracellular fluid.

In both cardiac and renal edema the extracellular fluid increased. Expansion of the extracellular fluid is generally accepted in the presence of edema, but recently attention has been directed to the abnormalities of the intracellular fluid. Elkinton[17] pointed out that the intracellular content of water and electrolytes may be abnormal in congestive heart failure. Talso[18] and his associates stated that edema is characterized by an increase in total tissue water representing an increase in both the extra- and the intracellular water content, while Friedberg[19] reported that intracellular dehydration develops in edema. It was seen in this report that the intracellular fluid slightly increased in cardiac edema and renal edema except

nephrosis. The lower value of the percentage of the intracellular fluid to the total body water obtained in this report does not necessarily signify the decrease of the actual intracellular fluid, because it is supposed that the body water is increased to a greater extent in the extracellular fluid than in the intracellular fluid. In nephrosis the extracellular fluid was markedly increased, while the intracellular fluid was decreased. This suggests that edema of nephrosis is different from cardiac and nephritic edema in the distribution of body water.

The edema fluid was lost mostly from the extracellular fluid when edema disappeared by administration of mercurial diuretics or cation exchange resin, and the percentage of the intracellular fluid to the total body water was larger than the normal even after the disappearance of edema (No. 5 b in Table II, No. 3 b in Table III). No. 4 and 5 in Table III also showed that the edema fluid was at first lost from the extracellular fluid in occurrence of diuresis. It is supposed from these facts that the restoration of the intracellular abnormalities is not enough to follow the shift of water from the extracellular space. This intracellular abnormalities in edema may be known from the higher thiocyanate-mannitol space ratio than the normal. Thiocyanate penetrates into cells in a normal state and its volume of distribution does not represent accurate extracellular space.[3)4)] Further, the cellular permeability to thiocyanate is markedly increased in pathologic states.[20)21)] The thiocyanate-mannitol space ratio was 1.18 on the average, that is, the thiocyanate space was about 20% larger than the mannitol space in normal subjects. This ratio was higher in the presence of edema than the normal, and did not return to normal even after the disappearance of edema, showing an increased cellular permeability to thiocyanate. If the increased cellular permeability to thiocyanate is an expression of intracellular abnormalities in edema, a higher value of the thiocyanate-mannitol space ratio may be considered as one of the criteria for intracellular abnormalities.

Summary

1. The antipyrine space (the total body water), the thiocyanate space and the mannitol space (the extracellular space) were simultaneously determined in 9 normal subjects and 12 edematous patients.

2. The extracellular fluid amounted to about 20% of the body weight or 35% of the total body water, and the intracellular fluid to about 35% of the body weight or 65% of the total body water in normal subjects. The extra- and intracellular fluids were more closely correlated with the total body water and the standard body weight than the actual body weight.

3. In cardiac and renal edema both the extra- and intracellular fluids increased, while in nephrosis the intracellular fluid decreased.

4. The edema fluid was derived first from the extracellular fluid and the shift of the intracellular fluid did not follow the decrease of the extracellular fluid in recovery from edema.

5. The thiocyanate-mannitol space ratio averaged 1.18 (range 1.13 to 1.22) in normal subjects. The higher value of thiocyanate-mannitol space ratio which is seen in edematous patients is considered as a criterion for intracellular abnormalities in edema.

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Inunction Method of BCG Vaccination, an Observation on the Schoolchildren applied with Lanoline-Silica Sand-Pilocarpine-BCG or Unguent Hydrophil-Silica Sand-Pilocarpine-BCG Vaccine

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We have contrived what we call the inunction method of BCG vaccination, in which we use neither lancet nor any kind of needle. The inunction method is to rub the mixture of BCG and some ointments into the skin.

According to our previous studies[1)2)] the BCG in the ointment vaccine can pass through rabbit skin into the body, and it is possible to vaccinate schoolchildren with BCG by means of inunction of lanoline-BCG or pilocarpine-lanoline-BCG vaccine without any local complications, although the rate of tuberculin conversion into positiveness is not so satisfactory.

In these experiments we made an effort to increase the rate of tuberculin conversion, using silica sand or unguent hydrophil.

Experimental

Preparation of the BCG Vaccines

1) Lanoline-silica sand-pilocarpine-BCG vaccine

The suspension of freeze-dried BCG was mixed with three times as much anhydrous lanoline and pilocarpine so that we got lanoline-1% pilocarpine-80 mg. BCG vaccine. Furthermore silica was added to the vaccine in the weight of 40 per cent.

2) Unguent hydrophil-silica sand-pilocarpine-BCG vaccine

The vaccine was prepared in the same way as described above.

3) Thick BCG suspension in physiological saline solution

This 80 mg. vaccine was used for scarification as a control.

4) Thin BCG suspension in physiological saline solution.

It was 0.4 mg. vaccine and was used for intradermal method as a control.

Vaccination

We vaccinated about 2300 tuberculin-negative or -doubtful children.

Inunction method. Every child smeared ca. 0.03 g. of one of the 1) and 2) vaccines on his own skin on the flexor surface of the left forearm and rubbed it with his right forefinger about 100 times, until the skin spot became congestive. Bathing was prohibited on that day and the next one.

Scarification method as the control. Six lines measuring about 1 cm. were scarified by means of lancet on the outside of left upper arm.

Intradermal method as the control. On the flexor side of the left upper arm 0.1 cc. of the 4) vaccine was injected intradermally at one point.

Local Complications and Regional Lymph Nodes Affection

We inspected the vaccination spots and regional lymph nodes two months to one year after the vaccination, three to four times at each school.

The inunction method provoked no local changes, with the exception of faint pigmentation observed infrequently. There was no bleeding as noted directly after the scarification and intradermal injection.

The scarification brought about a slight linear scar but no more complications.

The intradermal vaccination caused a scar in almost every case, and sometimes abscess, ulcer, crust or induration.

No regional lymph nodes were palpable in any series.

Tuberculin Reaction

We injected 0.1 cc. old tuberculin diluted 1 :2000 intracutaneously on the flexor side of the left forearm and measured crosswise the redness of the reaction about 48 hours after the injection, getting arithmetic average and deciding as follows:

Below 4 mm.	Negative.	Negative
5–9 mm.	Doubtful.	
Above 10 mm.	Positive	

Primary vaccination. The inunction of the lanoline-40% silica sand-1% pilocarpine-80 mg. BCG vaccine provoked the positive conversion of tuberculin reaction at the rate of 27.4% (43/157) two months after the vaccination. The intradermal vaccination as the control showed 65.4% positiveness (78/119) two months after. Six, nine or twelve months after the vaccination the tuberculin reaction remained positive respectively in ca. 5% in the inunction group, and respectively in ca. 40% in the intradermal one. The vaccinated children were 4–6 years old.

Re-vaccination. The re-vaccinated children were observed, being divided into two groups, that is a younger (7–8 years of age) and an elder group (9–11 years of age).

In the case of the younger group, the foregoing vaccination having been intradermal, 65.9% (163/247) became tuberculin-positive 2 months after the vaccination by the inunction of the lanoline-40% silica sand-1% pilocarpine-80 mg. BCG vaccine, and about 25% remained tuberculin-positive respectively 6, 9 or 12 months after the inunction. The intradermal re-vaccination as the control demonstrated 62.0% positiveness 2 months after the vaccination, and about 60%, unchanged, respectively 6, 9 or 12 months after.

In the case of the elder group, the foregoing vaccination having been intradermal, the rate of tuberculin conversion into positiveness was 85.8% (145/169) two months after the vaccination by the inunction of the lanoline-40% silica sand-1% pilocarpine-80 mg. BCG vaccine, and about 50% respectively 6, 9 or 12 months after, as shown in Table I. The intradermal re-vaccination as the control showed 72.9%, 56.5%, 84.6% or 76.6% positiveness respectively 2, 6, 9 or 12 months after the vaccination. The scarification as the control provoked the positive conversion of tuberculin reaction at the rate of 73.6%, 56.5%, 37.1% or 20.2% respectively 2, 6, 9 or 12 months after.

Another experiment was carried out in order to compare the usefulness of lanoline with that of unguent hydrophil for the inunction method. Vaccinating about 500 children, we could find higher positiveness of tuberculin reaction in the unguent hydrophil-BCG vaccine than in the lanoline-BCG vaccine.

Discussion

Previously Oike *et al.*[2] reported that the rate of tuberculin conversion was not so satisfactory in the inunction of the lanolin-BCG or the lanoline-pilocarpine-BCG vaccine. They concluded that the BCG in the vaccines

TABLE I

Tuberculin Reaction caused by Innuction of Lanoline-40% Silica Sand-1% Pilocarpine-80 mg. BCG Vaccine in Case of Re-Vaccination, Previous Vaccination being Intradermal

Vaccination method		Inunction	Intradermal	Scarification
2 months later	Children vaccinated	169	203	76
	Positive reactors	145	148	56
	Conversion rate	85.8%	72.9%	73.6%
6 months later	Children vaccinated	192	235	76
	Positive reactors	97	133	43
	Conversion rate	50.5%	56.5%	56.5%
9 months later	Children vaccinated	178	137	94
	Positive reactors	96	116	35
	Conversion rate	53.9%	84.6%	37.1%
12 months later	Children vaccinated	178	90	74
	Positive reactors	91	69	15
	Conversion rate	51.1%	76.6%	20.2%

passed into the body through the microscopic lesions of skin which were gotten by rubbing the skin at the time of inunction. The silica sand was added this time to the lanoline-pilocarpine-BCG or unguent hydrophil-pilocarpine-BCG vaccine for the purpose of getting more microscopic lesions of skin at the inunction. It was an attempt to heighten the percentage of positiveness of tuberculin reaction more than in the previous study.

The added silica sand answered this purpose anyway. In the primary vaccination by means of the inunction of the silica sand-vaccine we got 27.4% positiveness, which was higher than in the previous experiment using the vaccines containing no silica sand. In the re-vaccination the difference between this experiment and the previous one was strikingly obvious. In this experiment the rate of the positiveness was 65.9—85.8% two months after the vaccination and 22.2—51.5% twelve months after, although in the previous experiment it was 53% about two months after and 13% about one year after.

The duration of the positiveness of tuberculin reaction was also longer in the silica sand-vaccine than in the previous vaccine.

This time the unguent hydrophil-vaccine was better than the lanoline-vaccine in converting tuberculin reaction into positiveness. Unguent hydrophil has an affinity to water as lanoline does, and so it will be absorbed through skin easily. The BCG in the ointment may pass through skin at

the same time as the passage of unguent hydrophil. That is why we tried unguent hydrophil.

As a whole in primary vaccination the inunction method gave less positiveness of tuberculin reaction than the scarification or the intradermal method. But in re-vaccination the inunction method stood second with the scarification third and the intradermal first in the rate of tuberculin conversion and the duration of tuberculin positiveness.

No local complication was observed in the inunction, though we found sometimes abscess or ulcer in the intradermal and to a less degree in the scarification.

Conclusion

1. We can vaccinate schoolchildren with BCG by means of the inunction method, in which neither lancet nor any kind of needle is used.

2. The addition of silica sand to the vaccine for the inunction is useful for heightening the rate of tuberculin conversion.

3. In BCG re-vaccination the inunction of the lanoline-40% silica sand-1% pilocarpine-80 mg. BCG or unguent hydrophil-40% silica sand-1% pilocarpine-80 mg. BCG vaccine can convert tuberculin reaction into positiveness at a higher rate than the scarification method can, but at a lower rate than the intradermal method can.

4. The inunction method still remains unsatisfactory in primary BCG vaccination.

5. Unguent hydrophil may be better than lanoline as the base ointment for the vaccine of inunction.

6. The inunction method is safe and simple, and has no complication at the inunction spots and regional lymph nodes.

References

1) Oike *et al.*, Rep. Res. Inst. Tbc. & Lep., Tohoku Univ., 1952, **4**, 45.
2) Oike *et al.*, Tohoku J. Exp. Med., 1952, **56**, 283.

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Recovery of Membrane Potential of Dog's Ventricle after a Long Exposure to Low Temperatures, a Preliminary Communication

By

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The membrane resting and action potentials of the dog's ventricular free wall were tested with particular reference to the possibility of its recovery after the tissue had been exposed for protracted periods to the low temperatures ranging from 1 to 3°C. Excised samples of ventricular free wall taken from freshly killed dogs and kept in cooled Ringer's solution for periods from 24 to 72 hours, were rewarmed to 37°C within 10 minutes and its membrane potential was examined.

After 24 and 48 hours' exposures all samples resumed the resting and action potentials identical to those prior to the cooling, and after 72 hours, majority of samples recovered as well. The result is illustrated in Fig. 1.

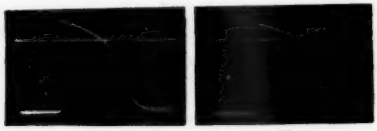

Fig. 1. Complete recovery of membrane resting and action potential of dog's ventricular muscle after exposure to low temperature (3°C) for 72 hours. Extracellular level of potential is indicated by the line with time scale (10 and 50 ms). Point on the bottom marks -100 mV.

The samples invariably showed spontaneous and repetitive firing of excitation (flutter or fibrillation) in the course of rewarming, but the automatism disappeared usually soon after the temperature had attained 37°C. Whether the specimen was kept cooled in oxygenated or deoxygenated Ringer's solution, it seemed to exert no apparent influence over the recovery of membrane potential thereafter.

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Chemistry and Biology of Lipids

XXI. On Group Lipids from Lungs of Group A and O Persons

By

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These group lipids were prepared in a state as pure as possible in a chemical way. The preparations showed no difference between the two groups in so far as examined.

Experiments and Comments

Preparation Procedure

First stage. Fresh human lungs from persons belonging to Group A or O were washed with water, freed from trachea and tracheoles mechanically, and after cutting into small blocks of about 5 ccm. size, preserved under 4.5 volumes of 91% denatured alcohol (containing 3.5% methanol). In seven years were collected lungs (parts) of 18 Group A persons and 10 Group O persons, and the blocks given were ground separately according to the blood group in a masticator and treated in a Faust apparatus, followed by drying in a vacuum desiccator (H_2SO_4) for 1 week. 1155 and 768 g. in total respectively of dry materials were obtained from 8600 g. fresh lung tissue of the Group A persons and 5053 g. fresh lung tissue of the Group O persons. The batches will be referred to as **Material A** and **O**.

Second stage. The materials were subjected to fractionation, taking the group activity as an indication, as will be illustrated on an example started with a 97 g. portion of Material A. The portion was powdered fine in a mortar and at first shaken with 3 changes of 5 volumes of dry acetone (Room temperature 10–15°C) for 8 days each to remove neutral fats and cholesterol, and then after drying in vacuo over H_2SO_4, freed from ordinary glycerophospholipids by extraction with dry ether. The remaining dark brown powder weighed 53 g. This was boiled with 5, 3 and 3 volumes of a methanol (dry)-chloroform (dry) mixture (3:1 by volume)[1] at 65–70°C for 40, 50 and 60 minutes respectively in succession. And the solutions united (dark red) were concentrated to one fourth the

volume by vacuum distillation and stood in a refrigerator at 0°C for 12 hours. The deposit (cerebrosides) dried weighed 30 mg. The mother fluid was further distilled in vacuo as far as possible, washed with acetone and dried (**Fr. Ia**). This substance was then taken up in 300 cc. of water-saturated ether. The insoluble part (300 mg. when dry) was discarded and the solution was also condensed to a syrup and washed with acetone. The brownish powder thus obtained (**Fr. Ib**, 288 mg.) was fractioned with chloroform, and the soluble part was recovered like as Fr. Ib. 258 mg. of a light brown powder was given—**Fr. Ic**.

Third stage. Since it was not yet homogeneous electrophoretically, a mixture of it and the corresponding fractions from other portions of Material A, which amounted to 1640 mg., was divided into 900 and 740 mg. parts (**Parts I** and **II**), and of the former, 60 mg. subdivisions were subjected to paper partition chromatography, employing a 80% phenol-dry chloroform mixture in the proportion of 4:1 by volume.

To describe the details, 60 mg. of the substance were dissolved in 0.3 cc. of water, and sampled 5 mm. wide and 25 cm. long on a 25×30 cm. square (with the shorter margins parallel to the fiber axis) of Toyo Roshi filter paper no. 2 along a horizontal line, ruled 3 cm. from the long side bottom. The shorter margins were tied together and the cylinder formed was stood in a dish containing 50 cc. of the organic mixture and covered with a bell of 17 cm. inner diameter and 33 cm. height. The chromatogram was developed at 22°± 1°C for 8 hours.

After drying, the paper was scissored at the height of Rf values of 0.7 and 0.3. The sections will be named *a*, *b* and *c* in the order from the top to the bottom. Section *a* contained the group substance and Sections *b* and *c* amino acids. The first section was irrigated in a descending way with chloroform (whereby the group-inactive contamination was removed) at first and then after thoroughly drying again, further irrigated with a mixture of methanol, chloroform and 80% phenol (4:4:1 by volume) until no more ninhydrin-positive substance came out. The second eluate was distilled in vacuo and the still-residue was washed with acetone and dried. It was taken up in chloroform. The insoluble part was centrifuged off, and the supernatant was distilled to dryness, washed with acetone and dried in vacuo over $CaCl_2$ to a constant weight—**Prep. A-I**. Yield 360 mg. (40% of Part I).

From the latter half (Part II) were obtained 290 mg. (33% of Part II) of **Prep. A-II** in a similar manner.

The crops (percentages) of preparations from Material O were resembling.

Properties of the Products

Anti-hemagglutinative potencies

The assay was carried out in test-tubes as described by Masamune and Akama[2].

As is shown in Table I, Prep. A-I was Group A active at a dilution of $1:1.6\times10^3$ and besides, Group B and Group O (against anti-OS saliva

TABLE I

Anti-hemagglutinative Potencies of the Intermediate and Final Products from Material A and of the Preparations from Material O

−: no agglutination, ±: agglutination was proved by means of a magnifier, +: agglutination could be seen with the naked eye. ++: marked aggl., +++: strong aggl. Anti-OS: anti-OS saliva chicken serum, Goat: normal anti-O goat serum. The antisera were diluted to titre 16 before use.

Product	Red cells	Serum	Dilution of the substances (1:)							
			10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
i. The potencies of the intermediate products from Material A, Part I										
Fr. Ia	A	α	−	−	−	+	+	+		
	B	β	+	+	++	++	+++	+++		
	O	Anti-OS	+	+	++	++	+++	+++		
	O	Goat	+	++	++	++	+++	+++		
Fr. Ib	A	α	−	−	−	−	+	+		
	B	β	−	−	+	+	++	++		
	O	Anti-OS	−	+	+	++	+++	+++		
	O	Goat	±	+	++	++	+++	+++		
Fr. Ic	A	α	−	−	−	−	+	+	++	
	B	β	−	−	+	+	++	++	++	
	O	Anti-OS	−	±	+	+	+++	+++	+++	
	O	Goat	±	+	++	+++	+++	+++	+++	
ii. The potencies of the final products from Material A										
Prep. A-I	A	α	−	−	−	−	−	±	+	+
	B	β	−	±	+	+	++	++	+++	+++
	O	Anti-OS	−	−	+	+	+	++	++	+++
	O	Goat	+	+	+	++	++	++	+++	+++
Prep. A-II	A	α	−	−	−	−	−	±	+	+
	B	β	−	−	+	+	+	++	++	+++
	O	Anti-OS	−	−	+	+	+	++	++	++
	O	Goat	+	+	+	++	++	++	+++	+++
iii. The potencies of the final products from Material O										
Prep. O-I	A	α	−	−	+	+	++	++	+++	+++
	B	β	−	−	+	+	++	++	+++	+++
	O	Anti-OS	−	−	−	−	−	−	+	+
	O	Goat	−	−	−	+	+	+	+	++
Prep. O-II	A	α	−	−	+	+	++	++	+++	+++
	B	β	−	−	+	+	++	++	+++	+++
	O	Anti-OS	−	−	−	−	−	±	+	+
	O	Goat	−	−	−	+	+	+	++	++

chicken serum) active at dilutions of $1:10^2$ and of $1:2\times10^2$ respectively, although the compound is believed to have been originally capable of in-

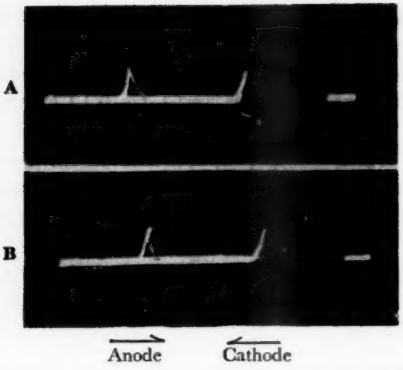

Fig. 1. Electrophoretic patterns of 0.5% solutions of Preps. A-I (**A**) and O-I (**B**) in veronal buffer pH 8.6, I 0.2; temp. 10°C; current 8 mA; exposure 60 min. after starting current. Ascending limbs to the left.

Mobility:

$$\text{Prep. A-I} \quad \frac{-1.00\times 0.10067\times 0.3}{0.008\times 3600} = -10.5\times 10^{-5}\ \text{cm}^2\cdot\text{volt}^{-1}\cdot\text{sec}^{-1}$$

$$\text{Prep. O-I} \quad \frac{-1.03\times 0.10067\times 0.3}{0.008\times 3600} = -10.8\times 10^{-5}\ \text{cm}^2\cdot\text{volt}^{-1}\cdot\text{sec}^{-1}$$

Table II

Solubilities of Preps. A-I and O-I

−: insoluble, ±: separingly sol., +: partly sol., ++: mostly sol., ###: perfectly sol.

Preparation	Solvent																	
	Ether		Petrol ether		Methanol		Ethanol		Glacial acetic acid		Benzene		Chloroform		Pyridine		Water		
	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Acidified*	Plain	Alkalinized†
A-I	−	−	−	−	±	+	±	+	+	+	###	###	###	###	###	###	+	++	###
O-I	−	−	−	−	±	+	±	+	+	+	###	###	###	###	###	###	+	++	###

* To pH 1.2 with HCl. The grade of solubility in 0.2 *N* acetate buffer pH 4.5 was the same. † To pH 9.6 with NaOH.

hibiting the agglutination of A erythrocytes alone (Cf. Table Ii). Similar relation was found to exist also between the native Group O lipid and preparations from Material O. It is assumed that, in some of the Group A lipid molecules, a physical or chemical link or links between the polysaccharide moiety and aglycon other than the N-glycosidic, stabilizing the

specific spacial configuration of Group A substances, have been scissored, and in some molecules of the Group O lipid on the other hand, a like bond or bonds, whose absence is one of the characteristic features of Group O substances, have been formed during the course of preparation (Cf. articles of Masamune and co-workers[2),3)]).

Electrophoresis

After electrophoresis at pH 8.6, the final products showed only one single boundary, as shown in Fig. 1. They gave no transparent solution on the acid side.

Soltbilities

See Table II.

Usual test-tube tests

The Group A and O substances behaved similar to each other, both reacting as follows: Molisch positive, biuret negative, indirect Osaki-Turumi negative, plasmal reaction after treatment with $HgCl_2$[4)] negative.

Tests for water-soluble components by paper partition chromatography according to Masamune and Hakomori[5)].

i) First and second stages. The example with Prep. A-I will be given. 25 mg. of the substance were weighed in a tube 11×2.5 cm. and boiled with a mixture of 2 cc. of dry methanol and 2 cc. of conc. HCl (12 *N*) for 6 hours under a return condensor with a ground joint. When cold, the hydrolysate was shaken with five 10 cc. portions of petrol ether. The petrol-ethereal solutions (upper layer of the successive mixtures separating on standing) were combined, washed repeatedly with water and distilled in vacuo, and 7 mg. of a yellowish gelatinous remainder were given which was set aside against need. The lower layer after shaking with petrol ether was distilled in vacuo to a syrup, taken up in 4 cc. of 6 *N* HCl and heated as above. The occurring humin was centrifuged off, and the brown supernatant was stood in vacuo over mixed solid sodium hydroxide and potassium hydroxide, adding a little water from time to time to expel off HCl as completely as possible. Finally the dried up residue was dissolved in 0.1 cc. of water and neutralized with 0.2 *N* NaOH to pH 7.8. This solution was subjected to the "first" and "second stage" chromatography.
a) First stage chromatography. 0.2 cc. portions were sampled, 5 cm. apart from the bottom, on slivers 2.5×60 cm. of Toyo Roshi filter paper no. 2. Solvents "I" (butanol saturated with water-phenol saturated with water-ethanol (5:3:1 by volume)-HN_3) and "II" (butanol saturated with water-phenol saturated with water-glacial acetic acid (5:2:1 by volume)) were used for irrigation. As indicators were employed 0.5% iodine in 95% alcohol and Dragendorff reagent for choline and 5% neutral lead acetate and H_2S for glycerophosphoric and inositolphosphoric acid. As is shown diagrammatically in Fig. 2**A**, choline and glycerophosphoric

acid were identified.

b) Second stage chromatography. 0.4 cc. portions were sampled at a corner of paper squares 30×30 cm. Irrigation solvents: primary run, butanol-acetic acid-water (4:1:2 by volume); secondary run, 80% phenol-NH_3. Indicator, ninhydrin. Spots corresponding to aspartic and glutamic acid, serine, glycine, hexosamines, arginine, ethanolamine, valine and/or methionine, phenylalanine and an unknown component were given (See Fig. 3**A**). None of those amino acids and amines could be detected when the preparations of the group lipids were chromatographed as such with 80% phenol, butanol-acetic acid (4:1 by volume) or butanol, proving all of them to be integrating constituents of the lipids but not contaminations.

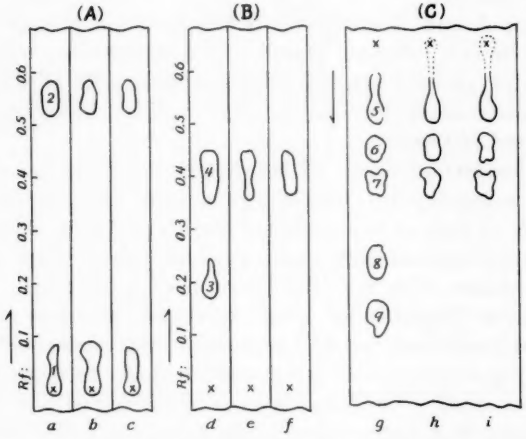

Fig. 2. First (**A**), third (**C**) and fourth (**B**) stage chromatograms of the water-soluble division of Preps. A-I and O-I developed by irrigation with Solv. II, the butanol-pyridine-water mixture and Solv. IV respectively. Temperature of test 24°±1°C. Irrigation ascending (Solvent fronts run 45 cm.) in (**A**) and (**B**), and descending (Duration 24 hrs.) in (**C**). Reference runs were carried out by sampling 0.02 cc. of 1% solutions with respect to the individual solutes.

a, d, g: reference run; b, e, h: Prep. A-I; c, f, i: Prep. O-I. 1 sodium glycerophosphate, 2 choline, 3 inositolphosphoric acid, 4 glycerophosphoric acid, 5 chondrosamine hydrochloride, 6 glucosamine hydrochloride, 7 galactose, 8 mannose, 9 L-fucose.

ii) "Third" and "fourth stages." 15 mg. of Prep. A-I were hydrolyzed with 3 cc. of 1 *N* H_2SO_4 at 100°C for 5 hours in a sealed tube, and after filtering off of the humin, freed from H_2SO_4 by addition of baryta to pH 6.8, followed by distillation in vacuo. The still-residue was dissolved in 0.2 cc. of water. a) Third stage chromatography. 0.07 cc. of the solu-

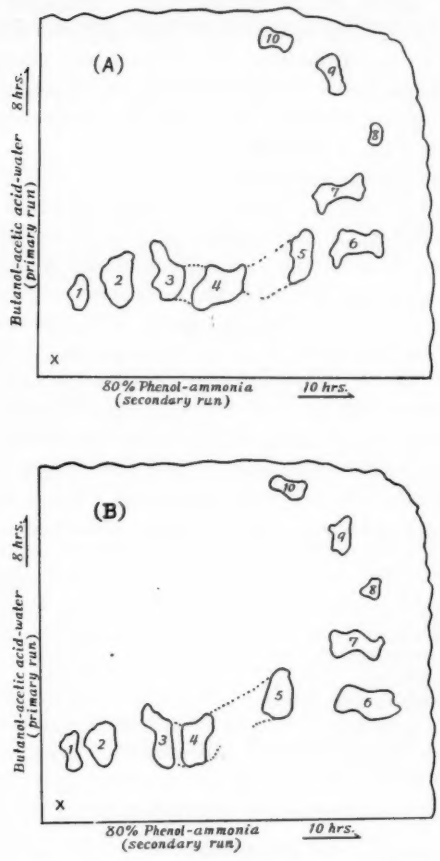
(A)
Butanol-acetic acid-water (primary run) 8 hrs.
80% Phenol-ammonia (secondary run) 10 hrs.
(B)
Butanol-acetic acid-water (primary run) 8 hrs.
80% Phenol-ammonia (secondary run) 10 hrs.

Fig. 3. Second stage chromatograms of the water-soluble components of Preps. A-I (**A**) and O-I (**B**). Temperature of test 24°±1°C.
1 aspartic acid, 2 glutamic acid, 3 serine, 4 glycine, 5 hexosamines, 6 arginine, 7 ethanolamine, 8 valine and/or methionine, 9 phenylalanine, 10 an unknown constituent.

tion was sampled on a sliver 7.5×60 cm. of Toyo Roshi filter paper no. 2. Irrigation solvent, butanol-pyridine-water (5:3:2 by volume). Indicator aniline hydrogen phthalate of Partridge. Galactose, glucosamine and chondrosamine were detected but neither of L-fucose and mannose in the chromatogram. See Fig. 2**C**.

b) Fourth stage chromatography. 0.04 cc. portions were sampled.

The size of the paper slivers, 2.5×60 cm. Irrigation solvents: 80% ethanol ("Solv. IV") and ethanol-methanol-glycerol-water (2:1:1:2 by volume) ("Solv. V"). Indicator, 5% neutral lead acetate together with H_2S. Glycerophosphoric acid was identified, as shown in Fig. 2**B**.

The same were the findings regarding the water-soluble components of Prep. O-I. See Figs. 2 and 3**B**.

Tests for water-insoluble components

Another 30 mg. portion of Prep. A-I was subjected to methanolysis with a mixture of 0.3 cc. of conc. sulfuric acid and 6 cc. of dry methanol in a 2.5×11 cm. tube by refluxing in a water-bath for 6 hours. When cold, the red-brown transparent solution was exhausted with changes of petrol ether. The petrol-ethereal solutions united (60 cc. in total) were washed well with water and distilled in vacuo to dryness. 7 mg. was the still-residue (**Div. I**). The H_2SO_4-methanol solution was next alkalinized with a methanolic KOH to pH 8.6 and the precipitate (K_2SO_4) centrifuged off. The supernatant was shaken with an equal volume of ether and the ethereal solution separating on standing was washed with water, dried with $CaCl_2$ and distilled by means of a jet pump to dryness. 6 mg. was the remainder (**Div. II**). The divisions gave the following consequences of tests.

Test	Division	
	I	II
Ninhydrin*	−	−
Alkaline permanganate*	±	+
Bromine (addition)	−	±
Fuchsine-sulfurous acid (Guyon)*	−	−
Hydroxamic acid-$FeCl_3$†	+	−
Diazobenzenesulfonic acid-NaOH (Rosenthaler)‡	−	++
$FeCl_3$ (Hantsch)§	−	−
Dragendorff		
Direct //	−	−
After treatment with dimethylsulfate ¶	−	−

* Spot test on paper. † Processed without prior treatment with $SOCl_2$. ‡ Test for OH groups. § Test for phenols and the alike. // N-methyl-sphingosine gives a yellowish orange ppt. ¶ Sphinogosine gives a yellowish orange ppt.

The positive hydroxamic acid-$FeCl_3$ reaction suggested an esterified fatty acid or acids, and the positive Rosenthaler a higher alcohol or alcohols. (Cf. Masamune *et al.*[6] and Hirata.[7]) The positive alkaline permanganate test might be ascribed to an unsaturated fatty acid or acids. The negative Guyon, ninhydrin and Dragendorff test disproved the presence of plasmals and free as well as methylated sphingosine.

Prep. O-I gave similar yields of the corresponding divisions and the same results of tests.

Quantitative analysis

Analysis gave the following figures (percentages).

Analysis	Preparation			
	A-I	A-II*	O-I*	O-II*
N†	3.4	3.4	3.3	3.3
P‡	1.7	1.9	1.9	1.9
Galactose§	1.0		1.1	
Ash //	10.6	11.0	9.7	8.7

* Prepared as was A-I. † Micro Kjeldahl, ‡ Haneda, Sinokawa and Yasuoka's modification of Plimmer method. § Masamune and Sakamoto[5]. // Pregl method without use of H_2SO_4.

Summary

1. Group A and O lipids were separated in an electrophoretically homogeneous state from human lungs of individuals belonging to respective blood groups, although group specificity became a little ambiguous during the course of preparation in accord with the group conversion *in vitro* of the pig lung group lipid which had been discovered by Masamune and Akama.

2. a) The preparations showed no discrepancies of solubility and N, P and galactose contents between Groups A and O.

b) The Group A and O lipids both contained aspartic and glutamic acid, serine, glycine, arginine, valine and/or methionine and phenylalanine as amino acids, and the hexosamines and galactose as sugars. Choline, glycerophosphoric acid, a fatty acid or acids and a higher alcohol or alcohols were also detected in the both group lipids.

c) Either of those group lipids was devoid of mannose, L-fucose, inositolphosphoric acid, plasmal and sphingosine.

In short, no chemical difference was found between these Group A and O lipids in so far as examined.

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H. Masamune

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4) Klenk & Thierfelder, Die Chemie der Cerebroside u. Phosphatide, Julius Springer Berlin, 1930, p. 76.

5) Masamune & Hakomori, J. Bioch. (Japan), 1952, **39**, **No. 5**, 5. An example, in which Masamune and Hakomori procedure was applied, is described in detail in an article of Masamune *et al.* (6)).

6) Masamune, Hakomori, Maehara & Suzuki, Tohoku J. Exp. Med., 1954, **59**, 231.
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Biochemical Studies on Carbohydrates

CLXXIX. Spectrophotometric Determination of Hexoses by the Aid of Thionalide

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Masamune and Ogawa[1] from this Institute published last year a spectrophotometric determination method of hexoses with thionalide. Since, however, the absorption was measured very inaccurately by means of a Spekker-photometer, namely with the naked eye, improvement of the method has been attempted according to the results of examination in a Beckman type spectrophotometer Hitachi. The renewed method is based on three reactions PHR_2, $SMHR_2$ and HR_{10} below, and is much more superior to the cysteine method of Dische *et al.*[2)2a)], who introduced the present analytical principle for sugar mixtures, because 1) the velocity of reaction is slow, that is, no hurried measurement of optical densities is requisite; 2) hexoses give more characteristic spectra; and 3) depressing effect of Cl^- on the color reaction is much less.

Analytical Procedure

A) PHR_2 (Primary hexose reaction after heating for 2 minutes). 0.5 cc. of a solution containing 5–50 γ of a hexose or hexoses as a whole or of a material in terms of total hexoses is pipetted into a 17×180 mm. test-tube and cooled in ice water for several minutes. 3 cc. of a mixture of 1 part of water and 7.5 parts of conc. H_2SO_4 (Junsei Chemicals, estra pure) by volume are added slowly along the wall with shaking under continuous cooling in the ice-water. The cold mixture is at first placed in water of 20–22°C for a few minutes and then in a vigorously boiling water-bath for exactly 2 minutes. It is cooled again in the ice water and thereafter warmed to 22–24°C in water, followed by further addition of 0.3 cc. of conc. H_2SO_4 containing 2.0 mg. of thionalide (Wako Pure Chemicals, pro analysi). The mixture here is shaken and stood in the bath of 22–24°C for 30 minutes, and the extinctions at 425 and 465 mμ wave-lengths are measured against the blank in the spectrophotometer to calculate the increment $D_{425}-D_{465}$.

B) $SMHR_2$ (Secondary hexose reaction after heating for 2 minutes in the presence of added mannose). Here the color reaction is processed with addition of mannose as was by Dische *et al.*[2] To 0.5 cc. of a solution similar to above of the unknown (in a test-tube of the same size as above) is added 0.2 cc. of a 0.05% mannose solution at first and then, under cooling, 4 cc. of the mixture of 1 part of water and 7.5 parts of conc. H_2SO_4 above. The mixture thus prepared is heated in a boiling water-bath strictly for 2 minutes, cooled and after further addition of 0.3 cc. of conc. H_2SO_4 containing 3 mg. of thionalide and following agitation, stood in an incubator at $22°\pm1°C$ for 72 hours to measure the absorption at 605 $m\mu$ wave-length or to measure the absorptions at 605 and 680 $m\mu$ wave-lengths, if a pentose or pentoses are simultaneously present in the unknown. The density D_{605} or the increment $D_{605}-D_{680}$ parallels the amount of total hexoses. In the blank and standard, mannose is also added extra to water or a hexose solution at starting.

C) HR_{10} (Hexose reaction by heating for 10 minutes). 1.0 cc. of a solution like the one above (in a test-tube of the same size as above) is added to with 3 cc. of a 19:45 water-conc. H_2SO_4 mixture by volume under shaking, and soon placed in water of 15°C. After about five more minutes in the water, it is further added to with 0.3 cc. of conc. H_2SO_4 containing 1 mg. of thionalide and shaken, then the mixture given is stood in actively boiling water for 10 minutes, quickly cooled under jet water and stood at room temperature. The reaction comes to completion rapidly even while being heated, giving an absorption spectrum which is constant during following 48 hours or even for several days. Extinctions at 380 and 410 $m\mu$ wave-lengths are measured to obtain the difference between them.

Calculation. The density value (or the increment) obtained in $SMHR_2$ and the density increments in the other reactions follow the law of Lambert and Beer. If the unknown contains three hexoses X, Y and Z, a solution of one of those sugars, for example, Z (a 10 mg% galactose solution is most expedient) is used as the standard, and the concentrations of the hexoses x, y and z in mg% are calculated by equations of Dische *et al.*[2]:

$$PHR_2 \quad x\frac{\varepsilon X_1}{\varepsilon Z_1}+y\frac{\varepsilon Y_1}{\varepsilon Z_1}+z=DR\ I\ \left(=\frac{\Delta D_1}{\varepsilon Z_1}\right)$$

$$SMHR_2 \quad x\frac{\varepsilon X_2}{\varepsilon Z_2}+y\frac{\varepsilon Y_2}{\varepsilon Z_2}+z=DR\ II\ \left(=\frac{\Delta D_2}{\varepsilon Z_2}\right)$$

$$HR_{10} \quad x\frac{\varepsilon X_3}{\varepsilon Z_3}+y\frac{\varepsilon Y_3}{\varepsilon Z_3}+z=DR\ III\ \left(=\frac{\Delta D_3}{\varepsilon Z_3}\right)$$

where DR I, II and III are optical density or density increment ΔD_1,

ΔD_2 and ΔD_3 for the unknown in the respective reactions divided by the corresponding values for the standard solution as converted into 1 mg% concentration εZ_1, εZ_2 and εZ_3. εX_n, εY_n and εZ_n (n=1, 2, 3) are $D_{425}-D_{465}$, D_{605} (or $D_{605}-D_{680}$) and $D_{380}-D_{410}$ values for solutions of X, of Y and of Z also as converted into 1 mg% concentration respectively. Average ratios (R_ε values) of the densities or density increments for various hexoses to the corresponding values for galactose, namely, $\frac{\varepsilon X_n}{\varepsilon Z_n}$ or $\frac{\varepsilon Y_n}{\varepsilon Z_n}$ are embodied in Table I. If two or only one hexose is present, two or one equation is sufficient.

TABLE I

Ratios of Densities or Density Increments (R_ε) for Various Hexoses to the Corresponding Values for Galactose in the Three Reactions

Hexose	R_ε		
	PHR_2	$SMHR_2$	HR_{10}
Galactose	1.00	1.00	1.00
Glucose	1.73	0.52	0.95
Mannose	0.81	0.086	1.18
Fructose	2.10	0.35	2.07

Explanation of the Procedure

1) In PHR_2, all hexoses give deep yellow colour at the 30th minute after addition of the thionalide reagent, giving a symmetrical absorption band with the maximum at 420 mμ wave-length. Pentoses get colored pale yellow with the absorption maximum at 292 mμ, but cause almost no absorption at the wave-lengths of 425 mμ and more. If the unknown contains a large amount of a pentose or pentoses, the latter are determined by another spectrophotometrical method, which will be published elsewhere, and the corresponding absorption at the wave-length of 425 mμ is subtracted from the total absorption given rise to by the unknown at the same wave-length for calculating hexoses. Methylpentoses remain uncolored and show almost no absorption within the range of wave-lengths from 350 to 465 mμ. Hexuronic acids give flat spectra and absorb a little of light at 425 mμ, although acetylhexosamines don't give rise to absorption at all at wave-lengths 425 and 465 mμ. And hence, materials containing a Goldschmiedt-positive mucopolysaccharide besides the Molisch-positive can be analyzed not so accurately as those which are free of this kind of mucopolysaccharide. This inaccuracy might be evaded only by separating off

of the Goldschmiedt-positive mucopolysaccharides prior to analysis. See Fig. 1 and Table II.

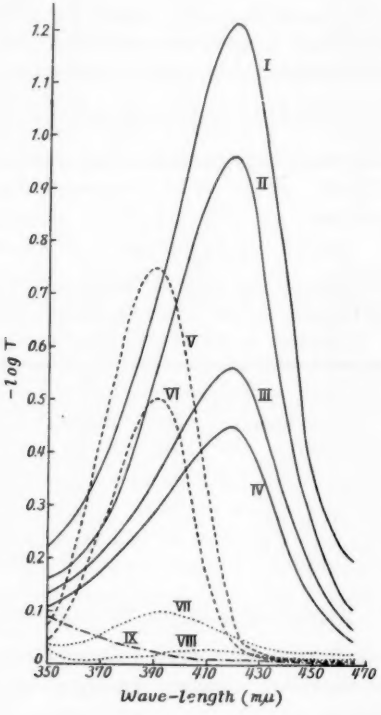

Fig. 1. Spectra in PHR_2 of 10 mg% solutions of various sugars. Cell 1 cm. thick.

I fructose, II glucose, III galactose, IV mannose, V ribose, VI L-arabinose, VII glycuronic acid, VIII L-fucose, IX N-acetylglucosamine.

When the reaction mixture is stood furtheron for 72 hours at 22–24°C (SHR_2), the spectra of individual hexoses change into those similar to the spectra shown in Fig. 2. (spectra by $SMHR_2$) possessing two peaks at 505 and 605 mμ (Cf. Table III), with change of the color nuance of the solution. By virtue of $D_{425}-D_{465}$ values in PHR_2 and $\frac{D_{595}}{D_{605}}$ values in SHR_2, one can check the result of paper partition chromatography of hexoses that is always advisable to preliminarily accomplish.

2) In $SMHR_2$ the optical density of the reaction mixture at the wavelength of 605 mμ is the total sum of the absorptions by the individual hexoses,

TABLE II

Densities at the Wave-lengths of 425 and 465 mμ for Solutions of Various Sugars in PHR_2 and Their Differences

Cell 1 cm. thick.

Exp.		Sugar		$D_{425}\times10^3$	$D_{465}\times10^3$	$(D_{425}-D_{465})\times10^3$
		Name	Conc. (mg%) in solutions			
I	a	Glucose	10	900	89	811
	b	"	8	750	81	669
	c	"	4	360	35	325
	d	Glycogen	10	1035	108	927
	e	Xylose	"	98	46	52
	f	Ribose	"	41	24	17
	g	L-Arabinose	"	20	12	8
II	a	Fructose	10	1210	203	1007
	b	Glucose	"	960	130	830
	c	Galactose	"	559	79	480
	d	Mannose	"	450	60	390
	e	L-Fucose	"	25	6	19
	f	Rhamnose	"	48	19	29
	g	N-Acetylglucosamine	"	12	10	2
	h	Glucuronic acid	"	50	16	34
	i	Galacturonic acid	"	51	25	26
	j	Sodium chondroitinsulfate*	"	26	10	16
	k	Yeast PNA†	40	77	60	17
III	a	Galactose	6	311	30	281
	b	Mannose	4	155	20	135
	c	Galactose+mannose	6+4	492	65	427
	d	Galactose+xylose	6+5	350	68	282
	e	Galactose+L-fucose	6+5	316	39	277
IV	a	Galactose	10	520	55	465
	b	Galactose+N-acetylglucosamine	10+20	520	50	470
	c	Barium sialate‡	10	25	10	15

* Prepared after Jorpes (Glucuronic acid 35%). † A preparation of E. Merck. Regarding its ribose content, see Masamune and Sakamoto[9]. ‡ Prepared as described in " Explanation of the Procedure ".

while, without addition of mannose, the absorptions are not additive. Pentoses cause a little absorption at the 605 mμ wave-length, but its influence upon the accuracy of hexose determination can be nullified by applying $D_{605}-D_{680}$ (See Fig. 2 and Table IV) to calculation of hexoses. The D_{605} or $D_{605}-D_{680}$ value increases slowly after 72 hours of standing of the reaction mixture at 22°±1°C but is regarded as practically constant within 24 hours.

3) In HR_{10}, hexoses give absorption bands extending over the wave-lengths from 340 to 410 mμ with the maximum at 383 mμ in glucose and at 380 mμ in other hexoses, with decreasing strengths in the order below from left to right, the last one causing the weekest absorption.

Table III

Densities at the Wave-lengths of 505 and 605 mμ for Various Sugars in SHR_2 and Their Ratios

Cell 1 cm. thick.

Fxp.		Sugar: Name	Sugar: Conc. (mg%) in solutions	$D_{505} \times 10^3$	$D_{605} \times 10^3$	$\frac{D_{505}}{D_{605}}$
I	a	Galactose	10	208	432	0.48
	b	Glucose	"	409	240	1.71
	c	Fructose	"	580	157	3.70
	d	Mannose	"	171	46	3.72
	e	Xylose	"	194	50	
	f	Ribose	"	65	16	
	g	L-Fucose	"	25	0	
	h	Glucuronic acid	"	20	14	
	i	Galacturonic acid	"	48	21	
II	a	Galactose	20	391	798	0.49
	b	"	12	248	495	0.49
	c	"	4	79	180	0.44
III	a	Galactose+glucose	16+4	540	770	0.70
	b	Mannose+glucose	16+4	489	195	2.51

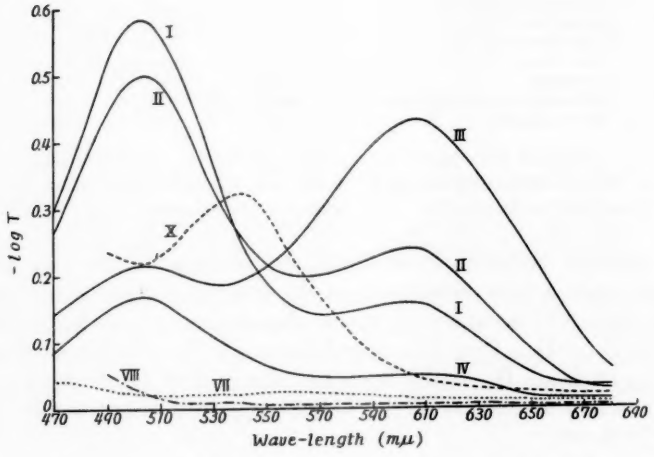

Fig. 2. Spectra in $SMHR_2$ of 10 mg% solutions of various sugars. Cell 1 cm. thick.

I fructose, II glucose, III galactose, IV mannose, VII glucuronic acid, VIII L-fucose, X xylose.

TABLE IV

Densities at the Wave-lengths of 605 and 680 mμ for Various Sugars in $SMHR_2$ and Increments between Them

Cell 1 cm. thick.

Exp.		Sugar		$D_{605}\times10^3$	$D_{680}\times10^3$	$(D_{605}-D_{680})\times10^3$
		Name	Conc. (mg%) in solutions			
I	a	Galactose	10	360	35	325
	b	"	5	185	16	169
	c	Glucose	10	188	15	173
	d	"	5	95	10	85
	e	Fructose	10	125	14	111
	f	Mannose	"	31	6	25
	g	"	5	19	3	16
	h	Glycogen	10	207	14	193
	i	Galactose+glucose	5+5	275	26	249
II	a	Galactose	20	680	90	590
	b	"	15	540	70	470
	c	"	10	360	28	332
	d	"	5	186	10	176
III	a	Xylose	10	40	22	18
	b	Ribose	"	13	3	10
	c	L-Fucose	"	0	0	0
	d	Galacturonic acid	"	19	10	9
	e	Glucuronic acid	"	13	8	5
	f	N-acetylglucosamine	"	0	0	0
IV	a	Galactose	10	377	49	328
	b	Galactose+N-acetylglucosamine	20	376	46	330
	c	Barium sialate*	10	0	0	0

* Prepared as described in " Explanation of the Procedure ".

Fructose>Mannose>Galactose<Glucose

All the hexoses give remarkably positive $D_{380}-D_{410}$ values. The corresponding density increments for methylpentoses are near 0, but those for pentoses are not negligible, in other words, the density increment for pentoses must be substracted from the increment for the unknown, after calculating from the pentose amount estimated otherwise.[3] The co-presence of hexuronic acids and acetylhexosamines does not impair the analysis, because D_{380} and D_{410} values equal each other in any of them (See Fig. 3 and Table V).

4) In the three reactions, sialic acid in such amounts as present in mucopolysaccharides is of no influence upon the determination of hexoses, as is illustrated in Tables II, IV and V. For the examination barium sialate (crude) was prepared as follows.

40 g. of bull submaxillary mucin isolated according to Blix[4] was boiled with 8 volumes of water in a current of N_2-gas at 110–120°C for 2 hours, and the paste given was dialyzed against changes of distilled water of below 10°C (8 *l.* in total). The dialysates were distilled at a low temperature to about 100 cc. under passing N_2-gas, and after draining through a cation exchanger (Amberlite IR-120) column, further distilled to 60 cc., whereby a precipitate occurred. The supernatant after centrifugation was alkalinized to pH 7.2 with the sat. $Ba(OH)_2$, heated on a boiling water-bath for 3 minutes and filtered while hot. The filtrate here was freed from excess barium with CO_2-gas and treated with active charcoal, then the centrifuged supernatant was distilled in vacuo to a thick solution and dried in a vacuum desiccator(H_2SO_4). The fragile brown substance given was powdered, and absolute methanol was added. And the turbid mixture was immediately precipitated with 10 volumes of acetone, washed with acetone and dried. The substance given was next exhausted with changes of abs. methanol, centrifuging off each time the part undissolved. The clear solutions were united, precipitated with acetone and dried in vacuo over $CaCl_2$. 1.7 g. of a white non-hygroscopic powder were yielded. Molisch, ninhydrin and Schiff (test for aldehyde) negative. Ehrlich (direct), Bial and Dische (diphenylamine reaction) positive. Ba 13.4%, N 3.2%, acetyl 16.2%, moisture 4.1% (The substance was dried to a constant weight in vacuo over P_2O_5 at 96°C). $[D]^{1cm}_{0.01\%}$ (Werner and Odin's method with *p*-dimethylaminobenzaldehyde[5]) =0.309 (at λ_{max} 555 mμ). $[D]^{1cm}_{0.01\%}$ (Werner and Odin's method with orcinol[5]) =0.972 (at λ_{max} 570 mμ).

Remarks

1) It is recommended to always determine the extinctions for a standard hexose solution in parallel with the main run, because the absorption for one and the same sugar solution varies more or less according to atmospheric humidity and temperature during the analysis.

2) By the reactions, the hexoses bound in polysaccharides can be estimated as accurately as free hexoses, that is, glycosidic linkages may be ignored as Dische[2)6)] assumed in their cysteine-sulfuric acid method. We ascertained it with glycogen and glucose with respect to extinction strengths at the mentioned wave-lengths, the relation between extinction strength and time of heating, the course of reaction progress after heating and the shape of resultant absorption spectra.

3) Preparations from tissues often contain non-sugar components which get colored when heated with sulfuric acid alone, that is, without thionalide. For such materials runs without thionalide (The solvent is used in place of the reagent) must be made to subtract the extinctions,

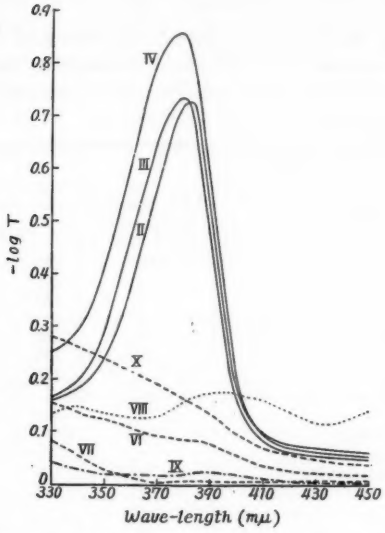

Fig. 3. Spectra in HR_{10} of 10 or 5 mg% solutions of various sugars. Cell 1 cm. thick.

II glucose (10 mg%), III galactose (10 mg%), IV mannose (10 mg%), VI L-arabinose (5 mg%), VII glucuronic acid (10 mg%), VIII L-fucose (10 mg%), IX N-acetylglucosamine (10 mg%), X xylose (5 mg%).

occurring thereby and measured against the blank for them, from the corresponding extinction values obtained by the ordinary procedure.

4) The thionalide reagents are comparatively stable and can be preserved for several days, when the vessel is well stoppered.

Factors Controlling the Intensity of the Reactions

1) Amount of thionalide. The extinctions increase with the increase of the amount of thionalide in PHR_2 in particular, and hence thionalide must be very accurately weighed for this reaction.

2) Concentration of H_2SO_4. In HR_{10} the extinctions for hexoses at the wave-length of 380 mμ is raised rapidly in different rates by increase of H_2SO_4 in the reaction mixture above the concentration chosen, and the relative absorption intensities are finally altered into the order of hexoses arranged below, mannose giving the least extinction, in other words, R_ε values are changed.

Fructose > Glucose > Galactose > Mannose

Therefore the H_2O-H_2SO_4 mixtures particularly that for HR_{10} must

TABLE V

Densities at the Wave-lengths of 340, 380 and 410 mμ for Various Sugars in HR_{10} and Increments between Those at the Last Two Wave-lengths

Cell 1 cm. thick.

Exp.		Sugar		D_{340} ×10³	D_{380} ×10³	D_{410} ×10³	$(D_{380}-D_{410})$ ×10³
		Name	Conc. (mg%) in solutions				
I	a	Mannose	10	295	862	90	772
	b	Galactose	"	200	740	89	651
	c	Glucose	"	179	728	86	642
	d	Xylose	"	525	341	160	181
	e	L-Arabinose	"	282	198	83	115
	f	L-Fucose	"	145	160	164	−4
	g	Rhamnose	"	158	165	190	−25
II	a	Ribose	10	392	220	110	110
	b	Glucuronic acid	"	97	64	43	21
	c	Galacturonic acid	"	83	10	10	0
	d	N-Acetylglucosamine	"	10	10	2	8
	e	Sodium chondroitinsulfate*	"	22	0	0	0
III	a	Fructose	5	507	1480	89	1391
	b	Mannose	10		902	109	793
	c	"	5		461	53	408
	d	"	2.5		237	24	213
IV	a	Galactose	5		430	58	372
	b	Glucose	5		418	62	356
	c	Galactose+glucose	5+5		840	116	726
	d	Galactose+L-fucose	5+5		510	165	345
	e	Galactose+L-arabinose	5+5		515	93	422
V	a	Galactose	10		769	115	654
	b	Galactose+N-acetylglucosamine	10+20		764	110	654
	c	Barium sialate‡	10		5	0	5

*, ‡ Signify the same as in Table II.

be exactly prepared.

3) Temperature. If, in $SMHR_2$, the reaction mixture is maintained at a higher temperature than 22°±1°C, for instance at 25°C, after addition of thionalide, the time till completion of the reaction is shortened and the ratios $D_{505}:D_{605}$ for hexoses become larger uniformly, although analysis is still performable if the extinctions given by a standard solution is taken advantage of. The constant extinctions continue for 1 day also in this case. If the temperature is lowered than 20°C, the reaction is slowed down enormously and analysis is made difficult.

4) Time of heating. In PHR_2 and $SMHR_2$ the maximum reaction occurs by heating for 2 to 2 1/6 minutes irrespective of the kind of hexose.

5) Size of test tube. In $SMHR_2$ and also SHR_2, where a long time

passes before completion of the reaction after addition of thionalide, the size of the test-tube influences the extinction at 605 mμ distinctly because of different quantities of moisture absorbed by the reaction mixture (The larger the inner diameter is, the stronger is the extinction).

6) Effect of NaCl. In PHR_2 as well as HR_{10} the presence even of 1/100 *M* NaCl is not harmful to the reaction (Table VI), but, in $SMHR_2$,

TABLE VI

Effect of NaCl upon the Reaction of a 10 mg% Galactose Solution

Cell 1 cm. thick.

Molarity of NaCl	PHR_2				$SMHR_2$		HR_{10}		
	$D_{420}\times10^3$	$D_{425}\times10^3$	$D_{465}\times10^3$	$(D_{425}-D_{465})\times10^3$	$D_{505}\times10^3$	$D_{605}\times10^3$	$D_{380}\times10^3$	$D_{410}\times10^3$	$(D_{380}-D_{410})\times10^3$
1/100	592	568	80	488	164	247	740	125	615
1/200	590	555	84	471	172	272	780	160	620
1/500	575	552	75	477	167	296	750	130	620
0	608	570	85	485	175	328	720	116	604

the partial reaction causing the absorption at the wave-length of 605 mμ is depressed by 17% by 1/200 *M* NaCl, while that causing the absorption at 505 mμ is not.

Through the Grant Committee for Scientific Researches, the Ministry of Education gave a grant, which enabled us to carry out this study.

H. Masamune

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Biochemical Studies on Carbohydrates

CLXXX. Spectrophotometric Determination of Pentoses in Sugar Mixtures and Polysaccharides

By

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(Received for publication, May 15, 1955)

Scrutiny of the procedures of Masamune and Ogawa for sugar analysis with thionalide has been extended, and regarding pentose determination[1] the modification below is recommended. Pentoses may be also determined besides hexoses by PHR_2 in the preceding article[2], if absorptions at the wave-lengths of 392 and 435 mμ are measured and stardardized with those for an appropriate pentose.

Analytical Procedure

A) PR-I (Pentose reaction I). 0.5 cc. of a solution containing 5–50 γ of a pentose or pentoses as a whole or of a material in terms of total pentoses is pipetted into a 17×180 mm. test-tube and stood in ice-water, and 3 cc. of a 7.5 : 1 H_2SO_4 (Junsei Chemicals, extra pure)-H_2O mixture by volume are added gently along the wall. The mixture given is shaken, cooled furtheron in the ice-water for a few minutes, and after warming to 22–24°C in a water-thermostat, heated in actively boiling water for 5 minutes. Thereupon it is again chilled by standing quietly in ice-water and added to with 0.3 cc. of conc. H_2SO_4 containing 1.0 mg. of thionalide (Wako Pure Chemicals, pro analysi), then the mixture here is shaken well and kept in an incubator at 22–24°C for 1 hour, followed by measurement of its optical densities at 392 and 435 mμ wave-lengths (This measurement must be finished quickly in summer time).

B) PR-II (Pentose reaction II). This is processed like as in A), but the H_2SO_4-H_2O mixture, with which the solution of the unknown is heated, is substituted with conc. H_2SO_4 (>95%). In the present case the heating is effected after tightly covering the tube-mouth with packed sheets of paraffin paper to avoid contact of the content with the steam from the boiling water-bath. The reaction mixture after addition of thionalide is stood in an incubator at 22°±1°C for 48 hours, and the extinctions at the wave-

TABLE I

Densities at 392 and 435 mμ Wave-lengths for Various Sugars in PR-I and Their Differences

Cell 1 cm. thick.

Exp.		Sugar		$D_{392}\times10^3$	$D_{435}\times10^3$	$(D_{392}-D_{435})\times10^3$
		Name	Conc. (mg%) in solutions			
I	a	Xylose	10	1100	46	1054
	b	"	5	576	25	551
	c	"	2.5	318	13	305
	d	"	1.25	168	8	160
	e	Ribose	10	530	15	515
	f	L-Arabinose	"	477	12	465
	g	Glucose	"	440	443	−3
	h	Galactose	"	230	220	10
	i	Mannose	"	185	180	5
	j	Yeast PNA*	40	568	13	555
II	a	L-Fucose	10	29	21	8
	b	Glucuronic acid	"	106	9	97
	c	Galacturonic acid	"	127	4	123
	d	N-Acetylglucosamine	"	4	0	4
	e	Sodium chondroitinsulfate†	"	43	13	30
III	a	Xylose	6	680	28	652
	b	"	4	426	20	406
	c	Xylose+glucose	6+8	1010	360	650
	d	Xylose+L-fucose	6+4	697	38	659
	e	L-Arabinose	6	306	15	291
	f	Xylose+L-arabinose	4+6	752	52	700
	g	Barium sialate‡	10	4	2	2

* A preparation of E. Merck. The ribose value obtained by the present method of analysis nearly matches the equivalent of the total purines. † Prepared after Jorpes (Glucuronic acid 35%). ‡ The specimen in the foregoing article[2]).

lengths of 390 and 432 mμ are measured.

Calculation. Density increments $D_{392}-D_{435}$ in PR-I and $D_{390}-D_{432}$ in PR-II are straight line functions of the amount of a pentose or total pentoses in accord with Lambert-Beer law (See Tables I and II). When only one pentose is present, the amount of that pentose is readily read from the standard curve, and if two or more are present, the total amount is expressed as one of those pentoses.

Explanation of the Procedure

1) By PR-I some pentoses get colored yellowish and some remain uncolored, but any pentose shows intensive absorption between the wavelengths of 350 and 430 mμ with the maximum at 392 mμ. The absorption increases rapidly with time after addition of thionalide. It becomes most intensive in 1 hour and then less and less gradually. There is seen no

TABLE II

Densities at 390 and 432 mμ Wave-lengths for Various Sugars in PR-II and Their Differences

Cell 1 cm. thick.

Exp.		Sugar		$D_{390}\times10^3$	$D_{432}\times10^3$	$(D_{390}-D_{432})\times10^3$
		Name	Conc. (mg%) in solutions			
I	a	Xylose	10	1310	74	1236
	b	"	5	650	36	614
	c	"	2.5	330	20	310
	d	"	1.25	170	12	158
	e	Ribose	10	460	51	409
	f	Glucose	"	690	701	−11
	g	Galactose	"	300	300	0
	h	Mannose	20	550	552	−2
	i	L-Fucose	10	32	30	2
II	a	L-Arabinose	10	393	26	367
	b	"	5	199	15	184
	c	Xylose	6	770	35	735
	d	Xylose+glucose	6+8	1210	482	728
	e	Xylose+galactose	6+8	995	265	730
	f	Xylose+monnose	6+8	952	235	717
	g	Xylose+L-fucose	6+6	820	65	755
III	a	Glucuronic acid	10	115	35	80
	b	Galacturonic acid	"	135	43	92
	c	N-Acetylglucosamine	"	4	0	4
	d	Sodium chondroitinsulfate*	"	50	26	24
	e	Barium sialate†	"	5	2	3

* The specimen in Table I. † The specimen in the foregoing article[2].

absorption at all at the 48th hour. Hexoses resemble pentoses in the respect of coloration, but their spectra are removed from those by pentoses, the maximum absorption occurring at 420 mμ, and besides, D_{392} and D_{435} equal each other. Almost no absorption is given rise to by methylpentoses and hexosamines. Hexuronic acids give spectra similar in type to those by pentoses. Although the absorption is much weaker, its influence upon the accuracy of pentose determination can not be neglected, so that the hexuronic acid-containing mucopolysaccharide, if present, must be separated off from the material (from an animal tissue) prior to analysis. See Fig. 1 and Table I.

2) Absorption spectra (peak at 390 mμ) similar to those by PR-I are given by PR-II of pentoses, but the reaction proceeds more slowly than in PR-I. The absorption at 390 mμ, which has increased to the utmost after 48 hours from the heating, remains practically unchanged for 12 hours, and several days pass before its perfect disappearance. As to their influence on the accuracy of pentose analysis by PR-II, hexoses which

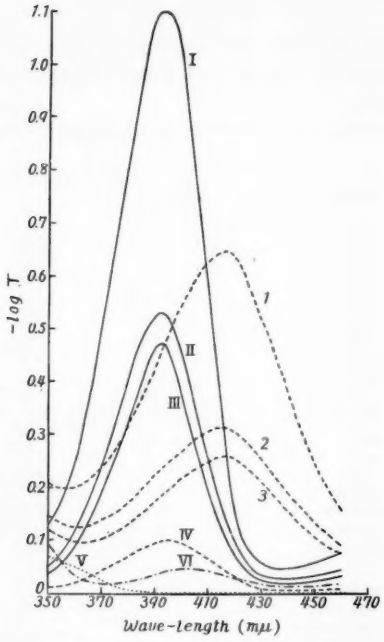

Fig. 1. Absorption spectra for 10 mg% solutions of various sugars in PR-I. Cell 1 cm. thick.
I xylose, II ribose, III L-arabinose, IV glucuronic acid, V N-acetylglucosamine, VI L-fucose, 1 glucose, 2 galactose, 3 mannose.

cause distinctly a symmetrical absorption with maximum at 415 mμ, methylpentoses and hexosamines, which are both almost no reactive, and hexuronic acids behave similar as described in 1). See Fig. 2 and Table II.

With respect to the intensity of maximum absorption in both PR-I and -II, pentoses are arranged in the order of

Xylose > Ribose > Arabinose,

arabinose being least productive of the absorbing derivative (Figs. 1 and 2).

3) Sialic acid is practically inert to the reagents.

Remarks

1) The blank, against which the extinctions are measured, is carried out by substituting water for the solution of material. On the other hand, a standard solution of a certain pentose is subjected to analysis each time

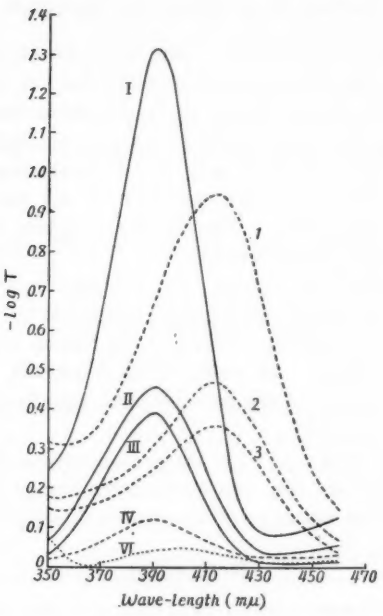

Fig. 2. Absorption spectra for 10 mg% solutions of various sugars in PR-II. Cell 1 cm. thick.
I xylose, II ribose, III L-arabinose, IV glucuronic acid, VI L-fucose, 1 glucose, 2 galactose, 3 mannose.

in parallel with the main run for the sake of accuracy in calculation.

2) The extinctions D_{392} and D_{435} in PR-I or D_{390} and D_{432} in PR-II are sometimes not equal to each other for hexoses. Therefore, to obtain most exact results, it is advisable to carry out a control run with galactose, besides the standard and blank, in parallel with the main in order to look for a wave-length between 430–436 mμ, where an extinction perfectly equal to D_{392} or to D_{390} is developed by that hexose, and apply the extinction at that wave-length instead of that at 435 or 432 mμ to calculation.

3) As in the analysis of hexoses,[2] another control run is requisite to make with conc. H_2SO_4 instead of the thionalide reagent for particular materials from tissues such as group lipids to subtract the extinctions hereby from those in the main, because those materials contain components giving products with H_2SO_4 showing absorption in the wave-lengths concerned.

4) The thionalide reagent must be used not immediately, but several minutes after preparation, because, if not, reproducible results are not obtainable.

Factors Controlling the Reactions

1) Concentration of H_2SO_4. One must be cautious regarding the concentration of the H_2SO_4 reagent especially for carrying out PR-II. If the sulfuric acid is less than 95%, the reaction in the second method is quickened entailing decrease of the extinction at 390 mμ already at the 48th hour after addition of thionalide with simultaneous occurrence of two absorption bands with respective maxima at 465 and 540 mμ, and the reaction mixture turns pink. The H_2SO_4 reagent should be naturally kept off from moisture, while preserved.

2) Temperature. In PR-II, the reaction mixture after addition of thionalide must be maintained pretty strictly at 22°±1°C, because the reaction velocity of the mixture is liable to variation with the temperature.

3) Time of heating. In either of PR-I and -II, the time of heating may be shortened even to 2 minutes without significant alteration of the extinction after the reaction.

4) Thionalide reagent. The larger the thionalide amount in the reagent is, the larger are the extinctions after completion of the reaction, although no change in the type of absorption spectra occurs.

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Biochemical Studies on Carbohydrates

CLXXXI. Spectrophotometric Determination of Methylpentoses in Sugar Mixtures and Polysaccharides

By

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Masamune and Ogawa[1] applied conc. HCl in the thionalide method for methylpentose analysis. Since its vapour damages the spectrophotometer, HCl has been replaced with H_2SO_4. The reaction by the improved method is more specific and reproducible, and besides, half or less the amount of the unknown taken in the former method is sufficient to obtain an accurate figure. Furthermore the method looks more preferable to Dische and Shettles'[2], because not only in the near-ultraviolet region but also in the visible is shown a characteristic absorpton for methylpentoses which serves for obtaining a reliable result.

Procedures of Analysis

A) MR-I (Methylpentose reaction I). 0.8 cc. of a solution containing 8 to 80 γ of a methylpentose or methylpentoses as a whole or of a material in terms of this kind of sugar is pipetted into a test-tube 17×80 mm. and cooled in ice-water, and 3 cc. of a 7.5 :1 conc. H_2SO_4-H_2O mixture by volume is added slowly along the wall, with following shaking under continuous cooling. When cold, the mixture given is placed at first in water of 22–24°C for a few minutes and then in a vigorously boiling water for 3 minutes. It is chilled sufficiently by quietly standing in the ice-water and again warmed to 22–24°C in water. Followingly it is further added to with 0.3 cc. of the H_2SO_4-H_2O mixture above containing 1 mg. of thionalide (See "Remarks"), and after thorough agitation, kept in an incubator at $22° \pm 1°$C for 24 hours to examine the extinctions at the wave-lengths of 400 and 430 mμ.

B) MR-II (Methylpentose reaction II). 0.6 cc. of a solution containing an amount mentioned of the unknown is chilled in ice-water, and mixed well with 3 cc. of a conc. H_2SO_4-H_2O mixture in the proportion of 45 :19 (by volume) under continuous cooling. Thereafter it is heated in

a boiling water-bath for 5 minutes, cooled in the ice-water, warmed to 22–24°C in water of this temperature and added to with the thionalide reagent above (See " Remarks ") in turn. The reaction mixture here is then stood at 22°±1°C for 24 hours as in MR-I. Extinctions are measured at the wave-lengths of 490 and 520 mμ against the blank.

Calculation. The amount of the methylpentose or methylpentoses as a a whole is calculated from density increments $D_{400}-D_{430}$ and $D_{490}-D_{520}$ respectively in MR-I and -II. The standard run is carried out in parallel with a solution of the methylpentose or one of the methylpentoses present in the unknown. L-Fucose and rhamnose give practically the same increments. Moreover, those increments are proportional to the amount of a methylpentose within the limits of 8 and 80 γ and of 6 and 60 γ respectively, following Lambert-Beer law.

Explanations of the Reactions

1) In MR-I some methylpentoses remain uncolored and some get colored faintly yellow, but any of them gives rise to strong absorption

TABLE I

Densities at the Wave-lengths of 400 and 430 mμ for Various Sugars in MR-I and Their Differences

Cell 1 cm. thick.

Fxp.		Sugar		$D_{400}\times10^3$	$D_{430}\times10^3$	$(D_{400}-D_{430})\times10^3$
		Name	Conc. (mg%) in solutions			
I	a	L-Fucose	10	1300	100	1200
	b	"	5	670	50	620
	c	"	2.5	350	25	325
	d	Rhamnose	10	1300	105	1195
	e	"	5	667	55	612
	f	"	2.5	320	20	300
II	a	L-Fucose	10	1290	110	1180
	b	Xylose	"	183	227	−44
	c	Ribose	"	78	90	−12
	d	Glucose	"	120	105	15
	e	Thymus sodium desoxyribonucleate*	20	63	82	−19
	f	Glucuronic acid	20	39	47	−8
	g	Galacturonic acid	20	90	110	−20
III	a	L-Fucose	5	680	41	639
	b	L-Fucose+glucose	5+10	820	177	643
	c	L-Fucose+galactose	5+10	762	130	632
	d	L-Fucose+xylose	5+5	746	134	612
	e	Barium sialate†	10	0	0	0

* The specimen of Masamune *et al.*[3] † The specimen in the article regarding hexose determination by the writers.[4]

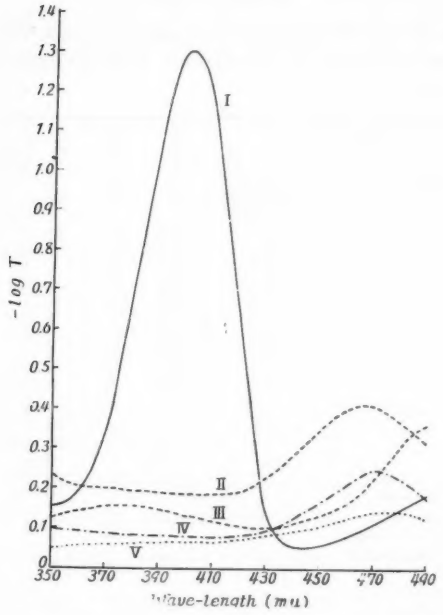

Fig. 1. Absorption spectra for various sugars in MR-I. Cell 1 cm. thick.

I L-fucose, II xylose, III glucose, IV ribose, V glucuronic acid. (All the sugars were examined as 10 mg% solutions excepting glucuronic acid (V), whose solution was 40 mg%.)

between the wave-lengths 350 and 430 mμ with the maximum at 400 mμ. The absorption becomes most intensive in 24 hours after addition of thionalide and remains practically constant for 12 hours, with subsequent gradual decrease till perfect disappearance after 5 days. By this reaction hexoses and pentoses change light red (or light violet) and pink respectively with appearance of an absorption band with the maximum at 500 mμ, but the differences between the extinctions at the wave-lengths of 400 and 430 mμ are neglectable as compared with the corresponding differences in methylpentoses. Desoxyribose and hexuronic acids change yellow and faintly red respectively whereas the absorptions occurring in the range of wave-lengths from 350 to 430 mμ are very weak with flat spectra. (Table I and Fig. 1)

2) In MR-II, methylpentoses show characteristic absorption spectra between the wave-lengths 430 and 520 mμ. The maximum absorption occurs at 490 mμ with deep yellow coloration of the reaction mix-

TABLE II

Densities at the Wave-lengths of 490, 520 and 575 mμ for Various Sugars in MR-II and Their Differences

Cell 1 cm. thick.

Exp.		Sugar		$D_{490}\times10^3$	$D_{520}\times10^3$	$D_{575}\times10^3$	$(D_{490}-D_{520})\times10^3$	$(D_{490}-D_{575})\times10^3$
		Name	Conc. (mg%) in solutions					
I	a	L-Fucose	10	1008	45	10	963	998
	b	"	8.3	816	37	7	779	809
	c	"	6.7	644	30	5	614	639
	d	"	5.0	468	21	4	447	464
	e	"	3.3	290	13	2	277	288
	f	"	1.7	112	5	0	107	112
	g	Rhamnose	10	945	42	7	903	938
	h	"	5	452	20	3	432	449
	i	"	2.5	203	9	0	194	203
II	a	Xylose	10	101	136	98	−35	3
	b	Ribose	"	89	116	79	−27	10
	c	Glucose	"	12	7	5	5	7
	d	Mannose	"	49	36	16	13	33
	e	Glucuronic acid	"	7	9	6	−2	1
	f	Galacturonic acid	"	25	26	18	−1	7
	g	N-Acetylglucosamine	"	1	0	0	1	1
	h	Sodium chondroitin-sulfate*	"	2	0	0	2	2
	i	Thymus sodium desoxyribonucleate†	40	23	23	13	0	10
III	a	L-Fucose	5	457	12	6	445	451
	b	L-Fucose+galactose	5+10	490	54	65	436	425
	c	L-Fucose+xylose	5+ 5	520	90	55	430	465
	d	Barium sialate‡	10	3	2	0	1	3

* Prepared after Jorpes (Glucuronic acid 35%). † The specimen of Masamune *et al.*[3] ‡ The specimen described in the article regarding hexose determination by the writers.[4]

ture, and is constant for about 1 week. Pentoses get colored light pink and show flat spectra with the peak at 550 mμ and the differences between the extinctions at 490 and 520 mμ are small. The differences, however, can not be ignored, if the amount of penotses as a whole is more than the equivalent of the total methylpentoses in the unknown so that, in such cases, the density increment $D_{490}-D_{575}$ should be taken for calculation in place of $D_{490}-D_{520}$ (Cf. Fig. 2). Hexoses cause no absorption in the range of wave-lengths from 420 to 580 mμ, while they colour faintly red or violet. Desoxyribose, sialic acid and hexuronic acids are scarcely colored and cause no recognizable absorption. (Table II and Fig. 2)

Remarks on the Procedures

1) As Dische and Shettles observed regarding their cysteine-H_2SO_4

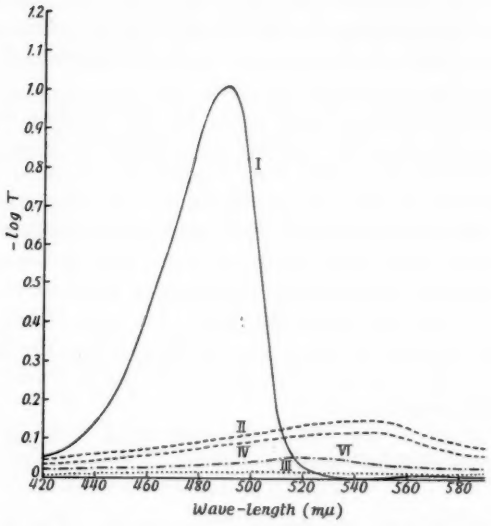

Fig. 2. Absorption spectra for various sugars in MR-II. Cell 1 cm. thick.

I L-fucose, II xylose, III glucose, IV ribose, VI galacturonic acid. (The sugars I–IV were examined as 10 mg% solutions and galacturonic acid (VI) as a 20 mg% solution.)

method, methylpentoses bound in polysaccharides containing also sugars of other kinds are analyzed just like free methylpentoses in sugar mixtures by the present procedures.

2) From the extinction values which have been obtained in either of the ways described must be often subtracted the corresponding values by a control run processed by substituting the conc. H_2SO_4-H_2O mixture (7.5 :1 by volume) for the thionalide reagent together with the blank of this run, because materials from tissues such as group lipids contain non-sugar components, which give rise to absorption at the wave-lengths concerned with the determination, when treated with sulfuric acid alone.

3) The thionalide reagent ought to be always freshly prepared and used immediately in MR-I and within 20 minutes in MR-II. If not, not only the strength of the absorptions but also the type of spectra changes in the cases of sugars other than methylpentoses particularly in those of hexoses, which impairs analysis.

Factors Controlling the Reactions

1) Amount of thionalide. In MR-I, increase, even doubling, of the

thionalide amount is accompanied by neither increase nor decrease of the extinctions for methylpentoses. But in MR-II, it entails decrease of the extinctions, for instance, the extinction at 490 mμ is lowered by about 20% when the thionalide amount is doubled.

2) Concentration of sulfuric acid. In MR-I, when the sulfuric acid concentration in the reaction mixture is elevated in excess, velocity of the reaction is decreased, and when the concentration is lowered, the reaction comes to completion earlier and absorption once developed disappears more quickly. In MR-II, increase of sulfuric acid in the reaction mixture above the concentration prescribed makes unstable the absorption after completion of the reaction, although no alteration of the analytical result ensues if the increase is restricted within the extent that conc. H_2SO_4 is replaced for the H_2SO_4-H_2O mixture as the solvent of thionalide. On the other hand, decrease of the sulfuric acid concentration in the reaction mixture results in decrease of the extinctions.

3) Temperature. In MR-I, the time which is required to complete the reaction after addition of thionalide depends markedly upon the temperature.

4) Time of heating and size of the test-tube. The time of heating (3 and 5 minutes) is in no need of strict adherence, because the extinctions at full development are not varied significantly thereby. Small difference in size of the test-tube also does not lead to erroneous results.

The Ministry of Education gave us, through the Grant Committee for Scientific Researches, a grant to defray the expenses of this work.

H. Masamune

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CLXXXII. Chemical Nature of KIK Factors
First Report: KIK Factors in Cancerous Gastric Juice*

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In 1937, Kozawa, Iwatsuru and Kawaguchi[2] discovered that cancerous gastric juices give rise to anemia in rabbits when injected intravenously, and named it KIK reaction. Since then, Iwatsuru and co-workers[3][4] continued the study of the reaction and stressed on its significance in diagnosis of gastric cancer, but the chemical nature of the substance or substances inducing the reaction remains still unestablished.

The writers fractioned cancerous gastric fluids by means of trichloroacetic acid, acetone and ammonium sulfate in turn, taking KIK reaction as an indication, and obtained two active preparations ("KIK factor I" and "II") which were electrophoretically almost homogeneous. The corresponding fractions of non-cancerous gastric juices were also separated. After examination of them it was concluded that there are two anemia-inducing principles in gastric fluids of stomach cancer patients and that one of them (KIK factor I) is a mucopolysaccharide and the other (KIK factor II) a mucoprotein.

Experimental

Preparation Procedure

Gastric juices were obtained by Dr. O. Ogata in Prof. Muto's surgical clinic from patients of gastric cancer and of non-cancerous diseases according to Katsch, and those, which were not stained with much blood or bile, were separately filtered through several cotton ganzes laid one over another and then sucked through a filter paper. The nearly clear filtrates were adjusted to pH 6.4–6.8 with NaOH or HCl, dialyzed against running water for 24 hours and distilled under reduced pressure to syrupy con-

* A half of the article, which was read before the 14th General Meeting of the Japanese Cancer Association, Kyoto, April 2-4, 1955[1], is corrected in part.

sistency, followed by drying in a vacuum desiccator over H_2SO_4. 0.2–0.3 g. of a powder was given from the gastric juice drawn out at one time. It contained usually about 30% ash. The following description concerns a fractionation example each of the batch of the dry powders obtained as above from cancerous gastric fluids and that of the powders from the non-cancerous.

1) *Fractionation of the cancerous material.* First stage. 4.0 g. of the dry material from the cancerous gastric juices were taken up in 100 cc. of water. The insoluble part was centrifuged off and washed with water, and the supernatant and washing united (130 cc. in all; green colored and turbid) were added to with half the volume of 10% trichloroacetic acid under agitation. The yellowish, flocculent precipitate was centrifuged after standing for half an hour, washed with three 10 cc. portions of 3.3% trichloroacetic acid at first and afterwards with 30 cc. portions of acetone and dried. 170 mg. (4.3% of the dry material) of a greenish, gray powder were yielded.—**Ppt. I.**

Second stage. The mother fluid and the 3.3% trichloroacetic acid-washing of Ppt. I were combined. A yellowish green, turbid fluid—**Fl. I**—was given. To it 1.5 volumes of acetone were added in portions. The white, ropy centrifugate was washed with changes of 60% acetone and the washings were added to the supernatant. This fluid mixture will be referred to as **Fl. II**. The centrifugate was further washed with acetone and dried. The powder given weighed 750 mg. (18.8% of the material)—**Ppt. II**. To Fl. II was added additional acetone to 80%. The flocculent precipitate here was separated in a centrifuge, washed with 80% and dry acetone and dried. 155 mg. (3.9% of the material) was the yellowish powder obtained—**Ppt. III**. The greenish, transparent mother fluid of Ppt. III was distilled under reduced pressure at 5–10°C to about 1/6 the volume in order to expel off most of the acetone, and the green precipitate that occurred hereby (36 mg.) was discarded. The clear fluid was dialyzed for 24 hours until the pH was elevated to 6.6 to remove most of trichloroacetic acid and distilled to 65 cc. To' the yellowish-greenish condensate, which was of pH 6.4, were added 3 volumes of acetone to 75%. And the yellowish flocculent precipitate was centrifuged, washed with acetone and dried. The powder given weighed 250 mg. (6.3% of the material)—**Ppt. IV**. The yellowish green, clear supernatant was added to further with as much acetone as to increase the acetone : water ratio to 10 : 1 (Acetone concentration became 91%), whereby a white fine flocculent substance was carried down. After standing for 1 hour, the precipitate was centrifuged, washed with three 30 cc. portions of acetone and with a 30 cc. portion of ether in sequence and dried. 534 mg. (13.4% of the material) was the yellowish powder yielded. It contained 23% ash—**Ppt. V**. The mother

fluid of Ppt. V was distilled *in vacuo* to syrupy consistency, precipitated with 50 cc. of abs. ethanol containing sodium acetate and dried after washing with ethanol and ether. A yellow powder amounting to 113 mg. (2.8% of the material) was given—**Ppt. VI**.

Third stage. Next, 500 mg. of Ppt. V were dissolved in 25 cc. of water (pH of the solution 6.4) and the yellow clear solution was saturated with ammonium sulfate by stirring up at 30°C with 18.6 g. of the salt. pH was lowered to 5.6. The flocculent precipitate was separated on a filter after 4 hours and then dissolved in water to reprecipitate by saturation with the same salt. Redissolution and reprecipitation was carried out once more.

a) The ammonium sulfate-saturated filtrates were united, diluted with 3 volumes of water and dialyzed against running water for two days, followed by vacuum distillation to 40 cc. The condensate was subjected to three repetitions of alternative shaking with a 10 g. (wet weight) of Amberlite IR-120 and with a 14 g. (wet weight) of Amberlite IRA-400, transferring by decantation from one vessel to the other, and after ascertaining no more occurrence of pH change with a new portion of those resins, filtered by means of a jet pump. The filtrate was free from NH_4- and SO_4-ions. The resins employed were washed in the order described with three 30 cc. portions of water. The filtrate and washings were distilled together *in vacuo* to a syrup, and precipitated with 50 cc. of ethanol and a few drops of ethanol saturated with sodium acetate. The deposit was washed with ethanol and ether and dried. An uncolored hygroscopic product was given—**Ppt. VII** (**"KIK factor I"**). Yield 60 mg. (1.6% of the dry material started with).

b) The precipitate by $(NH_4)_2SO_4$ was dissolved in 50 cc. of water and dialyzed against running water for two days, then the solution was distilled *in vacuo* to 30 cc. (pH of the condensate 6.4) and treated with the cation- and anion-exchange resins as above, followed by further condensation, precipitation and so on also similar to above. 120 mg. of a yellow powder were obtained—**Ppt. VIII** (**"KIK factor II"**). The crop is 3.2% of the dry starting material.

2) 5.2 g. of a dry green material from stomach juices of non-cancerous patients were fractioned just as in 1). Its fractions amounted as embodied on next page (The numbers of Ppts. signify the fractions corresponding to those of the same numbers in 1)). Figures in parentheses are the yeilds in percentages of the fractions above of the cancerous material.

Namely, as compared with the corresponding fractions of the cancerous material, the trichloroacetic acid-precipitate (Ppt. I) and particularly the fractions (Ppts. VII and VIII) corresponding to " KIK factors " were less and Ppt. II, the fraction richest in the group substances, was more in yield.

" Ppts." of the Non-cancerous Material (a 5.2 g. lot)

Ppt.	Yield	
	g.	in per cent of the dry material
I	0.12	2.3 (4.3)
II	1.51	29.0 (18.8)
III	0.51	9.8 (3.9)
IV	0.39	7.5 (6.3)
V	0.46	8.8 (13.4)
VI	0.071	1.4 (2.8)
VII	0.012	0.24 (1.6)
VIII	0.044	0.89 (3.2)

Properties of " KIK Factors " and the Corresponding Preparations from Non-cancerous Juices

The same fractions by several runs were mixed together to investigate their properties.

Animal experiment

The anemia-inducing effect was assayed by following Iwatsuru and Kato[4] in principle.

Various amounts of the fractions were dissolved in physiological saline and injected into the auricular vein of rabbits weighing 2–3 kg. The erythrocytes number of the venous blood was counted before and at intervals of 1 hour during 5 hours after the injection to determine the minimum dose per kg. body-weight, which caused 10% decrease of erythrocytes within that duration of time. In Table I are summarized the results on not only the final products but also the intermediate. The most part of this assay was carried out by Dr. O. Ogata, who will publish the results in detail elsewhere by himself.[5]

Electrophoretic examination

The substance from non-cancerous gastric juices, which corresponded to " KIK factor I ", was not examined because of shortage of material. Judging from their electrophoretic patterns (Fig. 1), the remaining substances looked nearly homogeneous in the present aspect, and on the other hand, " KIK factor II " and the corresponding fraction of the non-cancerous gastric juices did not coincide perfectly with each other as for the mobility.

Blood group potency

The hemagglutination inhibition test was processed by the procedure described by Masamune *et al.*[6] on a glass plate and the results are embodied in Table II. The faint A and B potencies of the preparations are most

TABLE I

Anemia-inducing Effect in Rabbits of Fractions of Cancerous and Non-cancerous Gastric Juices

Gastric juice	Fraction (Ppt.)	mg. substance injected per kg. bodyweight	Number of examinations	Decrease (%) of erythrocytes number*
Cancerous	I	1.0	3	10.2
	II	1.0	3	6.4
	III	1.0	3	3.8
	IV	1.0	5	14.2
	V	0.4	4	14.8
	VI	1.0	3	5.2
	VII ("KIK factor I")	0.4	2	17.5
		0.1	2	17.2
		0.05	5	15.2
		0.03	3	12.0
	VIII ("KIK factor II")	0.5	3	16.5
		0.3	3	13.4
Non-cancerous	I	1.0	3	3.5
	II	1.0	3	7.7
	III	1.0	3	8.5
	IV	1.0	3	6.3
	VI	1.0	3	3.7
	VII	1.0	3	2.4
		0.5	2	5.5
	VIII	1.0	3	5.0
		0.5	2	2.3

* Average decrease.

probably due to minute contamination by the blood group substances, most of which accumulated in Ppts. I–IV.

Solubility

Each pair of the substances from cancerous and non-cancerous gastric juices showed no discrepancy of solubility.

a) "KIK factor I" and the corresponding fraction of non-cancerous gastric juices. They were readily soluble in water of any pH value and pretty soluble in the saturated ammonium sulfate and the saturated sodium sulfate (33°C), but insoluble in glacial acetic acid, ethanol and ether. The watery solution was not precipitated by lead acetate, cupric chloride, barium acetate, baryta and trichloroacetic acid.

b) "KIK factor II" and the corresponding fraction of non-cancerous gastric juices. Insoluble entirely in the saturated ammonium sulfate, ethanol, acetone and ether and partly in the saturated sodium sulfate at 33°C. Soluble in glacial acetic acid and water of pH 1.0–9.6. Not precipitated from water by cupric chloride, barium acetate, baryta and trichloroacetic acid, but precipitated in part by lead acetate.

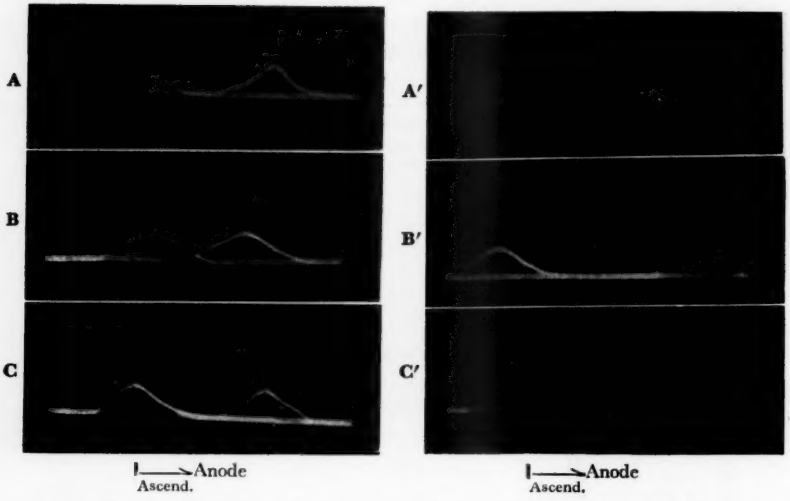

Fig. 1. Electrophoretic patterns of 1% solutions of "KIK factor I" and "II" and the mucoprotein from non-cancerous gastric juices corresponding to "KIK factor II" in 0.1 *M* veronal-HCl buffer of pH 8.6 and I 0.2 (adjusted with NaCl) (**A**, **B** and **C** respectively) and in 0.2 *M* acetate buffer of pH 4.6 and I 0.2 (**A′**, **B′** and **C′** respectively). Temperature 15°C; current 10 mA. Exposed 60 or 40 minutes after starting current (see below).

Mobility:

"KIK factor I" at pH 8.6, $\frac{(-0.2)\times 0.012029\times 0.3}{3600\times 0.01} = -2.0\times 10^{-5}$ cm^2·volt^{-1}·sec^{-1}

at pH 4.6, 0.0 (Exposure 40 minutes after starting current)

"KIK factor II" at pH 8.6, $\frac{(-0.5)\times 0.012029\times 0.3}{3600\times 0.01} = -5.0\times 10^{-5}$ cm^2·volt^{-1}·sec^{-1}

at pH 4.6, 0.0 (Exposure 40 minutes after starting current)

The non-canc. mucoprotein corresponding to "KIK factor II" at pH 8.6, $\frac{(-0.6)\times 0.011548\times 0.3}{2400\times 0.01} = -8.7\times 10^{-5}$ cm^2·volt^{-1}·sec^{-1}

at pH 4.6, $\frac{(-0.1)\times 0.006165\times 0.3}{2400\times 0.01} = -0.8\times 10^{-5}$ cm^2·volt^{-1}·sec^{-1}

(The same distance was travelled by the ascending boundary as by the descending in any of the cases.)

Test-tube tests

Each pair of the substances from cancerous and non-cancerous gastric juices behaved similar to each other toward test reagents excepting that "KIK factor II" showed the positive Millon reaction, though faint, contrary to the corresponding substance from non-cancerous gastric juices which gave the negative reaction. The results will be embodied in a table.

TABLE II

Group Potencies of "KIK Factors" and of the Corresponding Substances from Non-cancerous Gastric Juices

The sera (α and β sera, a normal anti-O goat serum and an anti-OS saliva chicken serum of respective titres 480, 480, 160 and 1280) were diluted to titre 16 for the assay. −: no hemagglutination, ±: faint hemaggl., +: slight hemaggl., ++: marked hemaggl., +++: strong hemaggl.

Substance		Red cells	Dilution of the substances (1:)							
Gastric juice	Fraction		Serum	10^2	2×10^2	$2^2\times 10^2$	$2^3\times 10^2$	$2^4\times 10^2$	$2^5\times 10^2$	$2^6\times 10^2$	$2^7\times 10^2$
Cancerous	"KIK factor I"	A	α	−	+	+	++	++	++	+++	+++
		B	β	−	+	+	++	++	++	+++	+++
		O	Goat-	+	++	++	++	+++	+++	+++	+++
		O	Anti-OS saliva	+	+	++	++	+++	+++	+++	+++
	"KIK factor II"	A	α	−	+	+	++	++	++	+++	+++
		B	β	−	+	+	++	++	+++	+++	+++
		O	Goat-	+	+	++	++	+++	+++	+++	+++
		O	Anti-OS saliva	+	+	++	+++	+++	+++	+++	+++
Non-cancerous	Ppt. VII	A	α	−	+	+	++	++	+++	+++	+++
		B	β	+	++	++	+++	+++	+++	+++	+++
		O	Goat-	++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS saliva	+	+	++	+++	+++	+++	+++	+++
	Ppt. VIII	A	α	−	+	++	++	+++	+++	+++	+++
		B	β	++	++	+++	+++	+++	+++	+++	+++
		O	Goat-	+	++	+++	+++	+++	+++	+++	+++
		O	Anti-OS saliva	+	+	++	++	+++	+++	+++	+++

Substance	Test				
	Molisch	Osaki-Turumi Indirect	Osaki-Turumi Direct	Neuberg-Saneyoshi	Lugol (for glycogen)
"KIK factor I"	+++	+	+	−	−
Ppt. VII of non-canc. gastric juices	+++	+~++	+	−	−
"KIK factor II"	++	+	+	−	−
Ppt. VIII of non-canc. gastric juices	++	+	+	−	−

Substance	Test				
	Biuret	Millon	Phosphorus	Bial	Dische
"KIK factor I"	±	−	−	+	−
Ppt. VII of non-canc. gastric juices	±	−	−	+	−
"KIK factor II"	+++ (red violet)	±	−	+	−
Ppt. VIII of non-canc. gastric juices	+++ (red violet)	−	−	+	−

Paper partition chromatography of the sugar and amino acid components

Sugar chromatography. i) Hydrolysis with hydrochloric acid and de-

scending chromatography was made at first according to the principle of Masamune *et al.*[6] Amounts taken of "KIK factor I" and the corresponding preparation from non-cancerous gastric juices were both 6 mg. and those of "KIK factor II" and the corresponding preparation from non-cancerous gastric juices were 11 and 10 mg. respectively. The hydrolysates were freed from humin and almost all of hydrochloric acid, and the dried up residues were dissolved each in 0.1 cc. of water (pH of the solutions here 3–5). A 0.04 cc. portion of the solutions were sampled excepting the case of the mucoprotein from non-cancerous gastric juices where 0.06 cc. was applied. The paper slivers (Toyo Roshi filter paper no. 2) used were 12×60 cm. and spotted with the solutions 10 cm. apart from the upper margin. Solvent for irrigation: butanol-pyridine-water (5 :3 :2 by volume). Indicator aniline hydrogen phthalate.

As shown in Fig. 2A, all the four preparations gave spots at the positions corresponding to chondrosamine, glucosamine, galactose, glucose, mannose and L-fucose and/or xylose in the order downward from above. The lowest spots had some red shade suggesting overlapping of the two sugars L-fucose and xylose.

ii) Next, 6.5 mg. of "KIK factor I" were hydrolyzed with 0.7 cc. of 1 *N* H_2SO_4 in a sealed tube at 100°C for 5 hours, and when cold, added to with the sat. $Ba(OH)_2$ to pH 5.8. The centrifuged supernatant and hot washings of the centrifugate were combined and evaporated in vacuo over $CaCl_2$ to dryness, and the remainder was dissolved in 0.06 cc. of water. Of the solution, 0.03 cc. was sampled on a paper sliver (5×60 cm.), which was then irrigated (also in the descending way) with a mixture of *n*-butylacetate, acetic acid, ethanol and water in the proportions of 3 :2 :1 :1 by volume. The chromatogram obtained (Fig. 2B) indicated a spot of xylose, though weak, besides the ones of hexosamine, glucose, mannose and L-fucose.

Amino acid chromatography. It was carried out in a usual manner. 6 mg. each of "KIK factor I", the corresponding fraction from non-cancerous gastric juices and "KIK factor II" and 5 mg. of the fraction from non-cancerous gastric juices corresponding to "KIK factor II" were hydrolyzed with 0.6 cc. (in the cases of the first three substances) or 0.5 cc. (in the case of the last one substance) of 6 *N* HCl in a sealed tube at 100°C for 24 hours. The humin was filtered off and washed. The filtrate and washings were evaporated together in vacuo over solid caustic soda to dryness and then liberated from HCl as far as possible by repeated evaporations with water. The final dry residue was taken up in 0.1 cc. of water. 0.02 cc. (in the cases of "KIK factors" and the substance from non-cancerous gastric juices corresponding to "KIK factor II") or 0.025 cc. (in the case of the remaining preparation) of the solutions

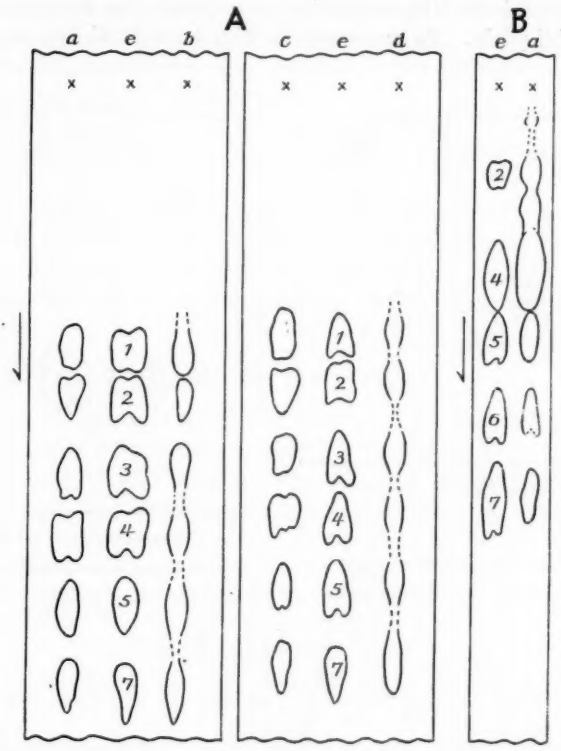

Fig. 2. Sugar paper partition chromatograms of "KIK factor I" and "II" and the corresponding substances from non-cancerous gastric juices developed by irrigation with butanol-pyridine-water at 23°±1°C for 22 hours (**A**) and that of "KIK factor I" by irrigation with *n*-butylacetate-acetic acid-ethanol-water (**B**) at 25°±1°C for 24 hours. Irrigation descending in the both cases. Indicator aniline hydrogen phthalate of Partridge. Reference runs were effected with a 0.02 cc. portion of 1% solutions with respect to individual sugars.

a: "KIK factor I", b: "KIK factor II", c: the mucopolysaccharide from non-cancerous gastric juices corresponding to "KIK factor I", d: the mucoprotein from non-cancerous gastric juices corresponding to "KIK factor II", e: reference run. 1: chondrosamine hydrochloride, 2: glucosamine hydrochloride, 3: galactose, 4: glucose, 5: mannose, 6: xylose, 7: L-fucose.

was sampled on a square 30×30 cm. of Toyo Roshi filter paper no. 2 for two-dimensional chromatography. Solvents for irrigation were butanol-acetic acid-water (4:1:2 by volume) for the primary run and 80% phenol (Ir-

rigation effected in an NH_3-containing atmosphere) for the secondary run. Indicator ninhydrin. As are shown in Fig. 3 (A, B, C, D), the four sub-

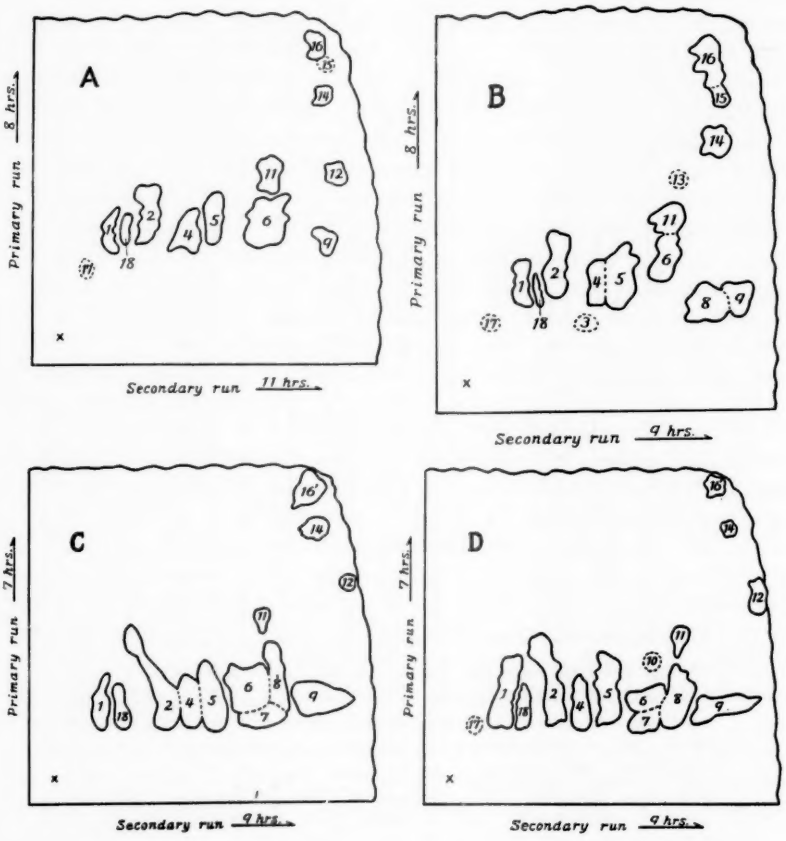

Fig. 3. Amino acid paper partition chromatograms of "KIK factor I" (**A**) and "II" (**B**) and Ppts. VII (**C**) and VIII (**D**) of the non-cancerous gastric juices. Temperature of irrigation 22°±1°C for the first two substances and 25°±1°C for the last two.

1: aspartic acid, 2: glutamic acid, 3: cysteine, 4: serine, 5: glycine, 6: hexosamines, 7: lysine, 8: histidine, 9: arginine, 10: threonine, 11: alanine, 12: proline, 13: tyrosine, 14: valine and/or methionine, 15: phenylalanine, 16: leucine and/or isoleucine, 16′: leucine and/or isoleucine and/or phenylalanine, 17: a peptide, 18: an unknown ninhydrin-positive component.

stances contained amino acids enumerated below besides those common to all: aspartic and glutamic acid, serine, glycine, arginine, alanine, proline, valine and/or methionine, leucine and/or isoleucine and an unknown ninhydrin-positive component.

"KIK factor I": phenylalanine, a peptide

"KIK factor II": cysteine, histidine, tyrosine, phenylalanine, a peptide

The substance from non-cancerous gastric juices corresponding to "KIK factor I": lysine, histidine, phenylalanine (?)

The substance from non-cancerous gastric juices corresponding to "KIK factor II": lysine, histidine, threonine, phenylalanine (?), a peptide

Quantitative composition

As shown in Table III, "KIK factor I" was less in L-fucose and sialic acid contents than the corresponding fraction of the non-cancerous gastric juices, and "KIK factor II" and the corresponding fraction of the non-cancerous gastric juices showed marked differences of analyses between themselves not only in percentages but also in equivalent ratios of the sugar components including sialic acid, although the kinds of sugar constituting the carbohydrate moiety of the four substances were the same.

Optical rotation in water

"KIK factor I": $[\alpha]_D^{15}=\frac{-0.64\times 1}{0.0101\times 1}=-63.4°$ (pH of the sol. 7.4)

"KIK factor II": $[\alpha]_D^{15}=\frac{-0.77\times 1}{0.0050\times 1}=-153.4°$ (pH of the sol. 6.8)

The non-canc. specimen corresponding to "KIK factor I": $[\alpha]_D^{25}=\frac{-0.302\times 1}{0.0110\times 1}=-27.4°$ (pH of the sol. 7.0)

The non-canc. specimen corresponding to "KIK factor II": $[\alpha]_D^{25}=\frac{-0.725\times 1}{0.0060\times 1}=-120.8°$ (pH of the sol. 7.0)

The substances from cancerous gastric juices were much more levorotatory than the respective corresponding substances from non-cancerous gastric juices.

Discussion

Needless to say, there remains a non-cancerous part of mucosa, whether large or small, in cancerous stomachs. Therefore "KIK factors" (preparations) are possible to have been mixtures of a KIK factor (pure) and the corresponding normal secretion, although "KIK factor II" proved pretty homogeneous electrophoretically probably due to the slight amount

TABLE

Analyses of "KIK Factors" and of the Corresponding

Analysis	In pee cent			
	Canc. gastr. juice		Non-canc. gastr. juice	
	"KIK factor I"	"KIK factor II"	Ppt. VII	Ppt. VIII
N*	6.4	11.2	6.1	13.4
Total hexosamines as glucosamine†	20.0	5.7	17.8	4.6
Galactose‡	6.3	2.9	5.4	1.3
Mannose‡	18.0	3.5	16.0	1.8
Glucose‡	15.2	1.9	15.8	1.2
L-Fucose§	2.5	1.1	5.6	1.4
Xylose//	1.8	0.9	1.5	0.5
Sialic acid¶	1.7	2.6	4.5	3.4
Ash**	3.8	1.7	6.1	4.1

* Micro Kjeldahl. † Total hexosamines were estimated without antecedent separa- & Sakamoto.[8] § A modification of Dische & Shettles.[9] // Masamune & Sakamoto.[10] use of H_2SO_4.

of the contaminating non-cancerous substance with somewhat different mobility. In other words, KIK factors (pure) must be assumed to rather show more discrepancy in various properties from the respective corresponding substances of non-cancerous gastric juice than "KIK factors" (preparations). Judging from the analytical figures above, KIK factor I (pure) and the corresponding non-cancerous substance are mucopolysaccharides and KIK factor II (pure) and the corresponding non-cancerous substance belong to the class of glucidamins in Masamune's terminology.

Summary

1. Two anemia-inducing substances ("KIK factor I" and "II") were prepared from gastric juices of stomach cancer patients. The corresponding substances were also separated from gastric juices of non-cancerous patients for comparison.

2. "KIK factor I" is assumed as a mixture of two mucopolysaccharides KIK factor I (pure) and the corresponding mucopolysaccharide secreted by the normal part of the gastric mucosa, and "KIK factor II" a mixture of two glucidamins KIK factor II (pure) and the corresponding mucoprotein secreted by the normal part of the gastric mucosa.

3. "KIK factor I" was much more effective than "KIK factor II" in causing anemia.

4. The substances from non-cancerous gastric juices were yielded

III

Substances from Non-cancerous Gastric Juices

In equivalents per equivalent weight			
Canc. gastr. juice		Non-canc. gastr. juice	
"KIK factor I"	"KIK factor II"	Ppt. VII	Ppt. VIII
4.10	25.15	4.39	37.33
1.00	1.00	1.00	1.00
0.31	0.51	0.30	0.28
0.90	0.61	0.89	0.39
0.76	0.33	0.88	0.26
0.14	0.21	0.34	0.33
0.11	0.19	0.10	0.13
0.05	0.26	0.15	0.43

tion of the both hexosamines by a minor modification of Blix method.[7] ‡ Masamune ¶ Werner & Odin (Direct Ehrlich)[11]; Calculated as $C_{11}H_{19}NO_9$.[12] ** Pregl without

much less, and were also less in anemia-inducing power or entirely devoid of this power. Furthermore, they diverged from "KIK factors" in quantitative composition and optical rotation.

5. "KIK factors" and the corresponding preparations from non-cancerous gastric juices possessed almost no blood group potencies.

This study was enabled to carry out by a grant from the Ministry of Welfare and Health, which is gratefully acknowledged.

H. Masamune

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1) Masamune, Sugimoto & Kawasaki, Gann, 1955, **46**, 126.

2) Kozawa, Iwatsuru & Kawaguchi, Folia haematologica, 1937, **57**, 251.

3) Iwatsuru & Kato, Gann, 1951, **42**, 45; Iwatsuru, Nippon Naikagakkai Zasshi (Jap.), 1952, **41**, 515; Iwatsuru, Kato, Taniguchi, Tamaki, Yutani & Kanagawa, Gann, 1952, **43**, 49; Iwatsuru, Kato & Yutani, *ibid.*, 1953, **44**, 17.

4) Iwatsuru & Kato, Gann, 1951, **42**, 51.

5) Ogata, unpublished.

6) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283. Regarding sugar paper partition chromatography, cf. also Mugikura, *ibid.*, 1955, **62**, 11.

7) Masamune & Yosizawa, *ibid.*, in press.

8) Masamune & Sakamoto, *ibid.*, this volume, p. 345.

9) L-Fucose was kindly analyzed by Dr. M. Sakamoto by Dische and Shettles' procedure

(J. Biol. Chem., 1948, **175**, 595) with modifications as follows.

0.8 cc. (instead of 1 cc.) of a solution of the unknown is taken in a test-tube, and after addition of 3 cc. (instead of 4.5 cc.) of the 6:1 H_2SO_4-H_2O mixture by volume, heated in a boiling water-bath for 10 minutes, followed by immediate chilling in ice water. The mixture was then further mixed with 0.1 cc. of a 3% cysteine hydrochloride solution, shaken and maintained at 25°C (water-thermostat) for 1.5 hours to measure extinctions at the wave-lengths of 400 and 430 mμ (not at those of 396 and 430 mμ) within subsequent 1 hour. By these minor modifications the reaction could be distinctly characterized for methylpentoses, namely, E_{400}-E_{430} values for even 10 mg% solutions of hexoses, hexuronic acids and acetylhexosamines became neglectable while larger extinction increments are utilized for methylpentose calculation, for instance, $[E_{400}\text{-}E_{430}]^{1cm}_{0.0025\%}$ for L-fucose amounts as much as 0.656.

10) Masamune & Sakamoto, Tohoku J. Exp. Med., this volume, p. 357.

11) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

12) Cf. Blix, Lindberg, Odin & Werner, Nature, 1955, **175**, 340.

Note at proof-reading: Ogata's article (5)) has been published in Tohoku Igaku Zassi (Jap.), 1955, **52**, 545.

The Tohoku Journal of Experimental Medicine, Vol. 63, No. 4, 1956

The Influence of Internally Used Sodium Thiosulphate upon the Oxygen Equilibrium of Hemoglobin

By

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The work of most of investigators[1)-5)] into the blood has proved that the oxygen equilibrium of hemoglobin is affected by the hydrogen ion concentration, by the carbon dioxide pressure, by the salts concentration and by the temperature of blood. From the time, when L. Pauling[6)] theoretically clarified the relationship between the molecular structure and oxygen equilibrium of hemoglobin, its biological function was more substantially connected to its chemical structure. In this connection we have experienced a new phenomenon which has not yet been reported. We observed a moderate cyanosis in a diabetic patient who had taken orally 3 g. of sodium thiosulphate a day for 2 months. At first we thought that this cyanosis was due to the formation of methemoglobin or other altered hemoglobin which could not transport oxygen. But this was not correct. The hemoglobin of this patient could completely be combined with oxygen by saturating the blood with pure oxygen but could not be fully oxygenated by saturating with air. Therefore, the affinity of the hemoglobin of this patient to oxygen should be reduced. On studying the blood of this patient we discovered that the effect of the dosed sodium thiosulphate upon the hemoglobin and oxygen equilibrium was greater than any other physiological factors ever known. Thus, we can shift the oxygen hemoglobin dissociation curve to the right by oral administration of sodium thiosulphate. The effect of sodium thiosulphate is not direct action upon hemoglobin. It is very interesting that we can affect artificially in vivo the dissociation curve of hemoglobin and oxygen without any side effect. To confirm the above fact we have made the following experiments.

Experimental

Methods and Materials

As subjects, we used our inpatients, and we administered 3 g. of

sodium thiosulphate a day. After a week or two, various degrees of cyanosis came to be discernible on these patients ranging from the slightest to the medium degree. At this stage we measured the oxygen saturation of arterial blood by van Slyke's method.[7] The most important matter in the circumstances, we must remember, is that the carbon monoxide capacity method has always to be adopted to decide the 100 per cent of oxygen capacity, because saturation with air produces a quite different result in this case. On having measured oxygen saturation of arterial blood, the dosage of sodium thiosulphate was stopped for a whole week, and then the same measurements were made again, of which the result is shown in Table I. In case cyanosis was remarkable, we measured the carbon monoxide capacity of the blood by saturating it with pure carbon monoxide gas and then we measured the oxygen capacity of the same blood by saturating with air, and of these we drew a comparison between the each. Furthermore, we measured the oxygen capacity by Drabkin's cyanmethemoglobin method[1] and drew a comparison of the above. (Table II) Next, after having measured the oxygen capacity saturating the blood with pure oxygen on one hand and with carbon monoxide on the other, we also drew a comparison of capacities between them. (Table III)

Further, to the normal blood was added the same quantity of distilled water on one hand and the same quantity of 1/100 N sodium thiosulphate on the other, and after several hours a comparison between carbon monoxide capacity of above two was drawn.

TABLE I

Oxygen Saturation of Hemoglobin of Arterial Blood before and during Cyanosis Produced by Sodium Thiosulphate Administration and after its Disappearance

Name of patient	Before	During		After
Kanno	96	91	87	96
Chiba	97	95	89	95
Nishida	95	89		
Kumada	94	88	88	94
Takeuchi		92		98
Minowa	94	89		93
Namba	97	93	87	
Wakatsuki		75	85	97
Matutani	95	91		96
Mean	95.4	88.5		95.6

TABLE II

O_2-Capacity Determined by Saturation with Air, CO-Capacity and O_2-Capacity Determined Spectrophotometrically by the Method of Drabkin, of the Same Blood of Patients who Showed Cyanosis by Thiosulphate Administration

Case No.	O_2-Capacity	CO-Capacity	O_2-Capacity determined photometrically
1	13.3		14.8
2	16.1		19.9
3	19.2	21.8	
4	13.9	15.4	15.8
5	13.5	15.8	
6	14.6	16.4	16.7
7	16.7	17.7	18.0

TABLE III

CO-Capacity and O_2-Capacity Determined by Saturation with Pure Oxygen of the Blood of Patients who Showed Cyanosis by Taking Sodium Thiosulphate

Case No.	CO-Capacity	O_2-Capacity
8	15.8	61.0
9	22.8	22.9
10	21.3	21.1
11	20.3	20.5
12	17.8	17.9

Results

As the Table I clearly shows, the oxygen saturation of arterial blood of the patients are lower while the sodium thiosulphate being dosed than before they begin their dosage. Moreover, the lowering is far below the physiological range. And the values measured after a week of free interval have regained the same normal value as measured before the dosage. By the experiments shown in Table I, it can be clearly seen that the hemoglobin of a patient while taking sodium thiosulphate does not fully saturate with oxygen under the normal alveolar oxygen tension. Therefore, we made experiments in saturating the blood with air. In spite of the fact that the hemoglobin of normal blood can be saturated almost completely under the oxygen pressure (about 150 mm. Hg) of the air, the hemoglobin of the patients does not combine with oxygen under the same oxygen pressure to the full extent, as shown in Table II. By this alone, however, it can not

be denied that a portion of hemoglobin may have become the derivative combining with carbon monoxide though it does not combine with oxygen. In fact the formation of sulfhemoglobin can not be excluded. But there is shown in Table III a fact that denies the above question. Though the blood of a patient with cyanosis can not be saturated under the atmospheric oxygen partial pressure (ca. 150 mm. Hg), it can perfectly be saturated with pure oxygen under the atmospheric pressure (760 mm. Hg). Table II proves that the hemoglobin content of the blood measured by carbon monoxide corresponds to the value yielded by measuring spectrophotometrically. On the above fact we assert that the cause of cyanosis is not the derivative which does not combine with carbon monoxide or pure oxygen.

Having gone through the above experiments, we realized that the hemoglobin of a patient under the administration of sodium thiosulphate shows diminished degree of affinity to oxygen. To study whether it is really due to the direct action of the sodium thiosulphate or not, we observed the comparison between the two oxygen saturations of hemoglobin: one is of the blood left alone after having been added the same quantity of distilled water and the other is of the same blood added same quantity of 1/100 *N* sodium thiosulphate solution. No difference between them, however, could be found at all.

Again, to testify if the oxygen dissociation curve of hemoglobin is affected by sodium thiosulphate immediately after its entering into blood in vivo or not, we compared the two arterial bloods: one taken 10 minutes after the intravenous injection of 20 m*l*. 10% solution of sodium thiosulphate and the other taken before the injection. The result is shown in Table IV. No difference can be seen in the oxygen saturations of hemoglobin of arterial blood before and after the injection.

TABLE IV

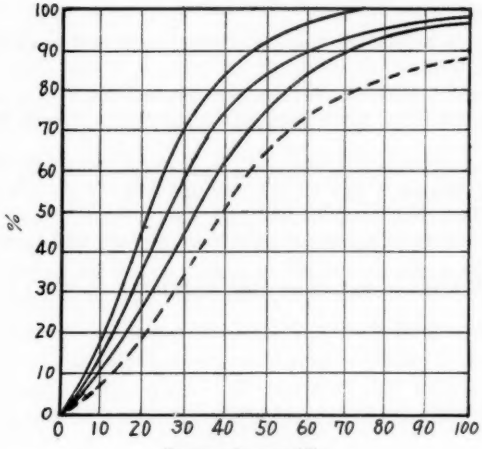
Oxygen Saturation of Arterial Hemoglobin before and after Intravenuous Injection of 10% Thiosulphate Solution

Case	Before	After
M.K.	94	94
S.G.	93	92
T.B.	95	94
O.S.	97	97
A.K.	98	98
H.T.	95	95
N.T.	97	97

Discussion

We proved that cyanosis gradually appears while the patients are being given sodium thiosulphate. By the above experiment, we confirmed that the cyanosis is not caused by the deterioration of the hemoglobin to another derivative which does not combine with oxygen, but by the decrease in affinity of hemoglobin to oxygen. The partial pressure of oxygen in the arterial blood is not considered to have been diminished in such a degree, because neither dyspnea nor symptom of oxygen lack can be seen. Accordingly this denatured hemoglobin shows the oxygen saturation about 89% at about 100 mm. Hg pressure of oxygen. We could not decide the oxygen dissociation curve of hemoglobin on this blood, but the dissociation curve as rough approximation should be supposed to be as shown in Fig. 1. The curve on the left side has been quoted from that decided by Henderson.

Fig. 1.



– – – Supposed oxygen hemoglobin dissociation curve of a patient showing cyanosis by administration of sodium thiosulphate. —— Dissociation curve taken from Henderson's data.

The figure shows that the dissociation curve shifts further to the right by sodium thiosulphate administration than the shift caused by the other physiological factors. The shift can not be considered as the direct action of sodium thiosulphate on the dissociation curve, from the point that the sodium thiosulphate added to blood has no effect at all. Moreover, no such change can be observed immediately after the intravenous injection of sodium thiosulphate. Through the above experiments, we are com-

pelled to decide that either the nature of hemoglobin or factors influencing its oxygen equilibrium may have gradually been changed after a definite duration by sodium thiosulphate administration. Anyhow, we can shift the oxygen hemoglobin dissociation curve to the right by artificial means. This fact may contain some significance not only in the theoretical view point but also in the practical application.

Summary and Conclusion

Patients orally given sodium thiosulphate 3 g. daily showed moderate cyanosis. The cause of the cyanosis was proved as oxygen unsaturation of hemoglobin of arterial blood. This unsaturation was due to the shift of oxygen hemoglobin dissociation curve to the right. Thus, one can decrease artificially the affinity of hemoglobin to oxygen in vivo without any discernible side effect.

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Critical Fusion Frequency of Flicker and the Electrical Excitability of the Retina

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Introduction

When the eye is exposed to intermittent light a sensation of flicker is produced, which, however, disappears at a certain critical frequency of intermittence, and this is known as the critical fusion frequency (CFF). The dependence of CFF on the intensity of illumination has extensively been investigated by a number of workers[1)-13)]. If the CFF is plotted as ordinates against the logarithm of intensity as abscissae a S-shaped curve is generally obtained at the fovea, but outside the fovea the relation is represented by two such curves which are interpreted as representing cone- and rod-functions respectively (Porter[5)], Hecht and Verrijp[14)-15)]). The linear portion of the S-shaped curve which is limited to the middle range of intensities is called sometimes " Ferry-Porter law."

The question as to whether the CFF is determined at the retinal level or at higher centers is not yet settled, although a number of experiments has been made, taking various kinds of phenomena such as ERG, action potentials of the optic nerve, of the optic radiation, and cortical potentials as the index.[16)-21)]

It is to be noted that the CFF is a relatively simple function of intensities, and that it is utilized as a measure of luminosity. Motokawa[22)] and Motokawa and Iwama[23)] showed that the increase of electrical excitability of the retina following an illumination may be represented by a S-shaped curve very similar to the curve for CFF, when plotted against the logarithm of intensities of pre-illuminating light. This fact suggests that there may exist some simple relation between the CFF and the ζ-value which is a quantitative expression of Motokawa's phenomenon. In the present experiment both kinds of quantities were measured on one and the same subjects under one and the same experimental conditions in order to compare their dependence on a variety of factors such as, intensity, retinal location, etc.

Experimental

Method

The method is described in detail in a previous paper[22], so that only the principle of the method will be mentioned here. The CFF was measured by a flicker apparatus in which flickering light was produced by means of a rotating sector of light-dark ratio 1 :1. The rate of flicker was measured by a well-calibrated electric tachometer attached to the apparatus. After a preliminary dark adaptation for about 20 minutes, the experiment was carried out without any background illumination. An opaque glass disc of 1 cm. in diameter was illuminated from behind by intermittent white light and viewed from a distance of 30 cm., so that the angle subtended by the disc at the eye was 2°.

The electrical sensitivity was determined following an illumination with fused light. The increase in sensitivity caused by the illumination was quantitatively expressed by ζ which is defined by the formula: $\zeta = 100\ (E-E_0)/E_0$, where E and E_0 denote electrical sensitivity measured with and without illumination. The electrical stimulus used was a single constant current pulse of 100 msec. in duration. A comparing procedure of Motokawa[24] was used when the subject told that a sensation of phosphene was uncertain.

Results

The ζ-time curve obtained at the fovea for fused white light of 52.5 lux is illustrated in the inset of Fig. 1. In this inset, percentage increases in electrical excitability of the eye over resting level are plotted as ordinates against times in seconds after termination of pre-illumination as abscissas. In this experiment, the velocity of the rotating sector was fixed just above the CFF and the left eye was exposed to white light for 2 seconds. The CFF in this case was 46 cycles per second (cps). As can be seen in this inset, the ζ-time curve has its crest at 2 sec., which will be called "the white process" (W-process).

In the first place, we studied the dependence of the magnitudes of the W-process and of the CFF upon the intensities of illumination. The data are shown in Fig. 1. In this figure, the magnitudes of the W- process or ζ and the CFF are represented by a broken and a continuous line, respectively. As can be seen in this figure, the CFF is related linearly to the logarithm of intensities over the range used (from -4 to $+4$ log units). The range of the CFF in this case was approximately between 100 and 6 cps. It is apparent that the Ferry-Porter law[4][5] holds good within the intensity range used in our experiment. The same is true of the magnitude of the W-process. According to the analysis by Motokawa, the W-process

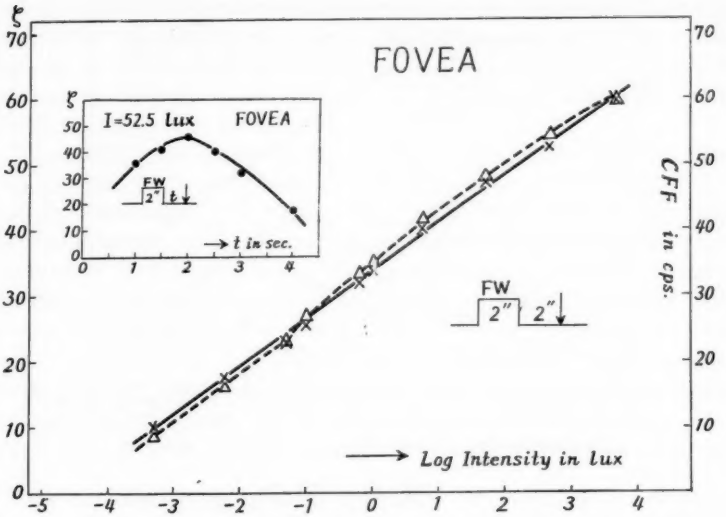

Fig. 1. Inset: Excitability curve obtained at fovea with pre-illuminating white light of 52.5 lux. The duration of pre-illumination used was 2 sec. Ordinates: ζ or percentage increases of electrical excitability over resting level. Abscissas: Time in second after end of pre-illumination. FW: Fused intermittent white light.

The dependence of magnitude of W-process (broken line) and of CFF (full line) obtained at fovea upon logarithm of light intensity. Ordinates: ζ or percentage increases of electrical excitability over resting level and CFF in cps.

having its crest at 2 sec. represents the excitation of cones. From the similarity concerning the dependence on intensities both CFF and W-process must be concerned with the cone-process at the fovea.

In the second place, a similar experiment was carried out in the peripheral retina. ζ-time curves obtained at 20° periphery are illustrated in Fig. 2. The pre-illuminating light used was varied, and the frequency of flicker was kept just above the CFF for each intensity. When such a high intensity as 525 lux was used, the curve indicated a single elevation at 2 sec., but at lower intensities two elevations having distinct maxima at 1.5 and 3 sec. appeared. This type of ζ-time curve is obtained from the periphery when the subject perceives a weak colorless sensation, irrespective of the wave-length of light. This process was designated " a twin-process " by Oikawa[25] and interpreted as concerning colorless sensations together with the so-called rod-process to be mentioned below.

We studied the dependence of the magnitudes of the two elevations

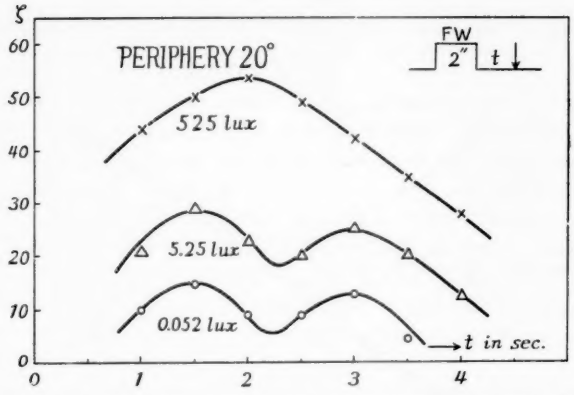

Fig. 2. Excitability curves obtained at periphery 20° for pre-illuminating white light of 2 sec. at various intensities of light. Number of each curve indicates the intensity of pre-illuminating white light in lux.

mentioned above upon the intensities of light in comparison with the CFF. The results are shown in Fig. 3. In this figure, the broken line and the full line indicate the magnitudes of the elevations at 1.5 and 3 seconds respectively, and the dotted line the CFF. As can be seen in this figure, the CFF gradually increases in the range of low intensities, but the rate of increase becomes steeper at about 0.1 lux and remains constant

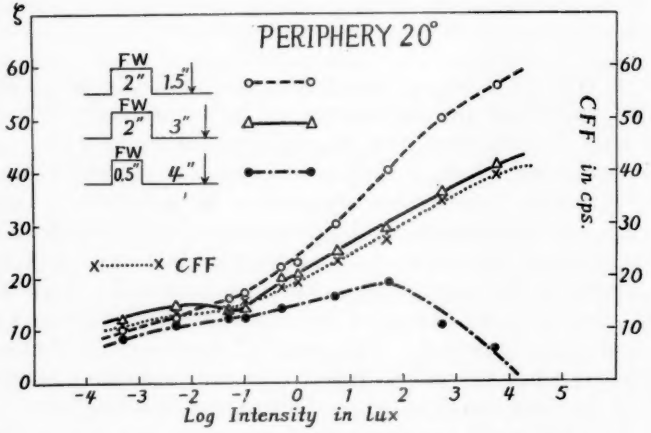

Fig. 3. The dependence of magnitudes of ζ at 1.5 sec. (broken line), ζ at 3 sec. (full line) and rod-process (chain line) and of CFF (dotted line) obtained at periphery 20° upon logarithm of light intensities.

above it. The CFF-log intensity curve apparently consists of two portions, as shown by previous investigators. As will be shown later, a similar change in the rate of increase occurs more earlier at 50° than at 20°. With regard to the ζ-log intensity curves for the magnitudes of the two elevations a similar tendency can be observed, too.

The chain curve at the bottom in Fig. 3 refers to the relation between the magnitude of the so-called rod-process and the light intensity. It was shown by Motokawa and Ebe[26] and by Oikawa[27] that a ζ-time curve shows a maximum at 4.5 seconds from the onset of illumination, and further Motokawa *et al.*[28] showed that the magnitude of this elevation varies with the wave-lengths of spectral lights just as the scotopic visibility curve. Therefore the process having a maximum at 4.5 seconds from the onset of illumination is called "a rod-process". When test light lasting 0.5 seconds as in the following experiment is used the rod-process is expected to appear having a crest at 4 sec. from the end of illumination. In Fig. 4 (c) the rod-process is shown together with the twin-process mentioned

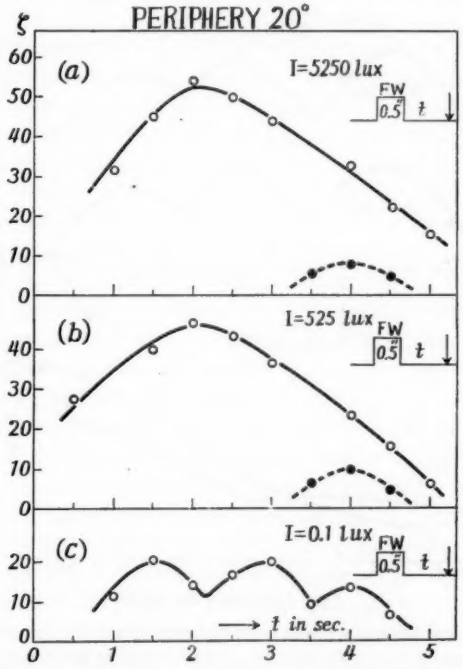

Fig. 4. Excitability curves obtained at periphery 20° with pre-illuminating white light of 5250, 525 and 0.1 lux. The duration of pre-illumination used was 0.5 sec. Broken lines represent rod- process.

above. At higher intensities of illumination the rod-process is generally masked by the W-process, but with special precautions, the masked rod-process may be disclosed, utilizing apparent thresholds[29] caused by rod-excitation. The broken curves shown in Fig. 4 (a) and (b) represent the rod-process thus disclosed. As is shown with the chain line in Fig. 3, the magnitude of the rod-process increases with increasing log intensity and reaches a maximum at about 50 lux, beyond which a further increase in intensity caused a decrease of the rod-process. The maximum ζ-value at this intensity is about 20. The fact that the magnitude of the rod-process decreases above a certain intensity, i.e. about 50 lux, may be due to an inhibitory action of cones upon rods, as was shown by Oikawa and Kurosawa.[30]

The behavior of the rod-process was observed at 50° from the fovea. The results are illustrated in Fig. 5. As can be seen in this figure, the

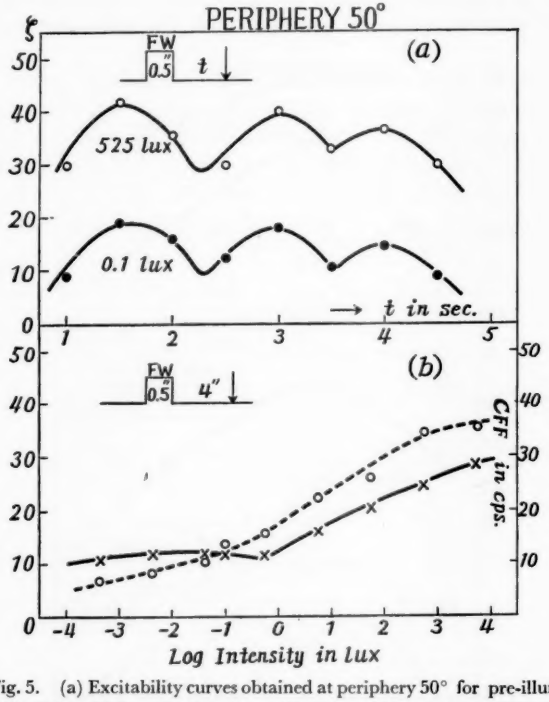

Fig. 5. (a) Excitability curves obtained at periphery 50° for pre-illuminating white light of 0.5 sec. in duration. Light intensities used was 525 and 0.1 lux respectively. (b) The dependence of magnitude of rod- process (broken line) and of CFF (full line) obtained at periphery 50° upon logarithm of light intensities.

curves for 525 and 0.1 lux are different in height, but they show three elevations having distinct maxima at 1.5, 3 and, 4 sec. respectively. With such a high intensity as 525 lux no such curve could be obtained at 20°, where a curve having a single maximum at 2 sec. was obtained, instead. Another difference is that the magnitude of the rod-process is decidedly greater at 50° than at 20° when the intensity of illumination is high. In consequence, as is shown in Fig. 5 (b), the magnitude of the rod-process obtained at 50° increases with increasing intensities and shows no such decline as observed at 20° over the range of intensities used. The CFF-log intensity curve consists of two clearly different portions (the full curve), while the magnitude of the rod-process is represented by a single smooth curve.

Discussion

Over the range of intensities of the light stimulus used, the CFF and the magnitude of the W-process increase hand in hand with the logarithm of illumination intensity, and the relation could be represented by a single straight line at the fovea. This fact may be so interpreted that the CFF and the W-process in terms of ζ refer to the function of the cones. However, when the peripheral area of 20° from the fovea was illuminated, a break in the curves occurred at about 0.1 lux. Von Kries[31)-32)], Hecht[33)] and others interpreted this fact in such a way that the two portions of the CFF-log intensity curve represent the functions of the rods and the cones, respectively. As was shown by Simonson *et al.*,[34)] the break may be explained by an abrupt change in the number and type of excited elements. According to their explanation, the lack of such a break in animals with one type of receptor cells or in central areas smaller than 2°, which contain only cones, seems an excellent corroboration of Hecht's duplicity theory. The fact that the break occurred at a higher intensity in the retinal area 50° from the center than in that 20° will be due to the higher degree of predominance of the rods over the cones.

For the interpretation of the ζ-log intensity curves obtained in the periphery some knowledge of ζ-values measured at 1.5 and 3 seconds from the end of illumination will be necessary. As shown by Motokawa[35)], 1.5 and 3 seconds are the characteristic intervals of the yellow and the blue processes respectively. Therefore the ζ-values measured in the range of high intensities, in which a photopic luminosity curve would be obtained, must be concerned with photopic vision.

On the other hand the twin-process which was discovered by Oikawa[25)] shows two maxima at 1.5 and 3 seconds, and subserves colorless sensations like the rod-process. Since the spectral sensitivity of the twin-process coincides with the scotopic visibility curve, it must represent a scotopic

process. In view of these facts ζ-values measured in the lower range of intensities may be considered to concern scotopic vision. Therefore it is not surprising that our ζ-log intensity curves obtained from the retinal periphery should consist of two parts just as the CFF-log intensity curve. This fact is in turn further evidence that ζ-values measured at 1.5 and 3 seconds can refer to photopic vision under photopic conditions, but to scotopic vision under scotopic conditions.

In contrast to the double structure of the curves mentioned above, the curve for the rod-process is a smooth S- shaped curve, showing no break anywhere, as is illustrated in Fig. 5.

The decline of the magnitude of the rod-process at higher intensities at 20° from the center may be interpreted as due to inhibition exerted by simultaneous cone excitation, and the absence of such a decline at 50° may be interpreted in connection with the fact that the number of inhibitors or cones is smaller in this region of the retina.

Summary

Using the method of electrostimulation by Motokawa and a flicker apparatus the effect of light upon the electrical sensitivity of the eye was studied in comparison with the critical fusion frequency (CFF) at the fovea and in the retinal peripheries. The light-dark ratio of flicker was 1 :1, and the frequency of flicker was held just above the CFF in the measurement of electrical excitability.

1. At the fovea the CFF increased linearly with the log intensity of illumination. The increase in electrical excitability following illumination showed the same linear relation to the log intensity as the CFF.

2. At periphery 20° the CFF- log intensity curve consisted of two parts, belonging to scotopic and photopic vision. A similar double structure could be observed in ζ-log intensity curves where ζ denotes the magnitude of increase in electrical excitability measured at 1.5 or 3 seconds from the end of illumination.

3. After the method of Motokawa the rod-process was measured in isolation. The magnitude of the rod-process versus log intensity showed no double structure. The curve increased with rising intensities till about 2 log units, and then decreased at 20°.

4. At periphery 50° there was no such decline in the magnitude of the rod-process. The decline observed at 20° was attributed to the inhibitory effect of the cones upon the rods.

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